FURTHER STUDIES OF FACTORS INFLUENCING THE GROWTH AND SURVIVAL OF RHIZOBIA IN HUMUS AND SOIL CULTURES.

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The undersigned hereby certify that they have read and recommend to the School of Graduate Studies for acceptance, a thesis entitled, "Further Studies of Factors Influencing the Growth and Survival of Rhizobia in Humus and Soil Cultures", submitted by J.F.T. Spencer, B.Sc., in partial fulfilment of the requirements for the degree of Master of Science.

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A B S T R A C T

Growth and survival of root nodule bacteria in soil and humus cultures were studied by means of plate counts and respirometric methods. Screw capped glass jars were more satisfactory than cardboard cartons for packaging of seed inoculation cultures and mineral oil added as a sticker and preservative was not detrimental to growth and survival. The effect of various inorganic nutrient elements and sucrose was also studied. In general, any particular element stimulated growth only if the level of that element in the soil used as a base was below optimum. Nitrate and sucrose stimulated growth when either was used alone, but both used together greatly depressed growth.

Respiration measurements on sterilized soils inoculated with Rhizobium meliloti showed that oxygen uptake was roughly proportional to soil organic matter content. The addition of plant residues greatly increased oxygen uptake, alfalfa having a greater initial effect than straw.

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TABLE OF CONTENTS

Table of Contents

INTRODUCTION

The function of the root nodule bacteria as agents of fixation of atmospheric nitrogen in symbiosis with leguminous plants has been known since 1886, and the value of leguminous crops to mankind has been known for many thousands of years. All of the ancient civilizations of the old and new worlds knew of legumes and used them in their agriculture. Even more primitive peoples such as the Swiss Lake Dwellers left evidence of the use of peas and beans among the ruins of their villages, evidence which was found long after they had vanished as a people.

As time went on the importance of legumes in any planned system of farming became more and more evident. From the time of the Romans onward, except for the time of the decline of all knowledge during the Dark Ages, anyone making a study of good farming practices was aware of the value of legumes as human food, livestock fodder and soil improver. However, it was not until the early part of the nineteenth century that the methods of scientific investigation were far enough advanced to be brought to bear on the problem, and it was not until after nearly one hundred years of investigation and sometimes acrimonious controversy that the difference, as regards nitrogen sources, between the Leguminosae and other plants, and the role of the root nodule organisms, was established.

Once it was definitely established that the microorganisms were necessary for the fixation of atmospheric nitrogen, research divided itself into two general classes. The first was the practical application of the new knowledge to farming practices; the second, pure research on the morphology, life history and biochemistry of the bacteria, including the formation and structure of the nodule, the relationship between host and bacteria, and the mechanism of the fixation process. Research on the practical applications at first outstripped that on the other phases, and in the early part of the twentieth century a most voluminous literature on the inoculation of legumes in the field, on different types of inoculation cultures, and on requirements for growth and survival of the bacteria, accumulated. Later on, after satisfactory seed inoculation cultures and methods of using them had been made available, work was concentrated more and more on the mechanism of the process of biological nitrogen fixation. As new chemical and physical methods of investigation became available, the intermediate steps in the fixation process have been made more clearly visible, until there can now be discerned the first vague outlines of possible mechanisms of fixation. However, the outlines have failed to stand out with a great deal of clarity, even in a general manner, and this has been especially true for

the symbiotic nitrogen fixing organisms.

Nevertheless, while the investigation of the fixation process has been carried on, work on the practical applications of the problems has been found necessary. The existing inoculation cultures, while vastly superior to some earlier ones which have appeared and been discarded, have been found to require improvement. The problems of contamination, of maintenance of the moisture content and of aeration have been found to be paramount in the production of an inoculum which would supply a sufficient number of viable organisms for good inoculation of the seed to which it was applied. In addition, the survival of the organisms from year to year under field conditions has been found to be of vital importance to perennial legume crops.

This investigation has been concerned with the development of an improved legume inoculation culture, which would maintain a large number of viable organisms longer, in a form convenient for handling. The study of factors influencing the growth and survival of the root nodule bacteria in these cultures has been continued, and a study of the growth and survival of these organisms in field soils has been started.

REVIEW OF LITERATURE

Since the discovery in 1888 by Hellriegel and Wilfarth, as reviewed by Fred et al (24) of the relationship between the nodules and the leguminous plant, and its bearing on the ability of legumes to fix atmospheric nitrogen, a voluminous literature on the subject has accumulated. Beijerinck, as reviewed by Fred et al (24) isolated the organisms responsible for root nodule formation in 1888, and since that time the mass of papers has become so great that a brief review of the entire subject is impossible. Fred et al (24) have reviewed the literature prior to 1932, and Wilson (85) has dealt with the biochemistry of symbiotic nitrogen fixation as it was understood in 1939. The literature on growth and survival of rhizobia, and the development and use of legume inoculation cultures has been reviewed by Hedlin (31) though his review deals largely with the older papers.

It is proposed here to review the papers published in the last twenty years insofar as is possible, referring to older papers only when necessary to preserve the continuity of the subject.

(1) Occurrence of Rhizobia in Soils

In 1930, Wilson (80) made a study of the seasonal variations in the numbers of Rh. leguminosarum and Rh.

trifolii in field soils. There was a wide variation in numbers of both species, depending on the treatments received by the various plots. On acid soils, numbers were as low as one in five grams of soil. On other soils, numbers rose as high as 100,000 per gram. In general numbers of Rh. trifolii were higher than numbers of Rh. leguminosarum especially on the acid soils. There was a fall in numbers of both species during the winter, with an increase in the numbers as the temperature rose in the spring.

Wilson (81) continued this study in 1931, using the same dilution method of counting, and again found that Rh. trifolii was present in larger numbers than Rh. leguminosarum. He was unable to relate this phenomenon to pH, moisture content or seasonal variation, except on the very acid soils, but thought there might be some connection with the type of inorganic salts present.

Walker and Brown (78) used Wilson's (80) dilution method to study the relationship between numbers of Rh. meliloti and Rh. trifolii and soil management practices. They found that:

(a) Numbers depended on previous cropping and fertilizer treatment history of the soil.

(b) Numbers were larger when clover or alfalfa was grown every third year than when legumes were not grown, and also where alfalfa had been plowed up a month previously.

(c) The condition of the soil with respect to organic matter, lime and phosphate had a greater influence on numbers of rhizobia than did frequency of growth of the legume hosts.

(d) Manure, limestone, crop residues or rock phosphate, alone, or especially in combination produced large populations of rhizobia.

(e) Recommendations for inoculations should take into account the cropping and soil management history of the land.

Thorne and Brown (63) continued these studies in 1937 and obtained similar results. They found that legumes promoted a more stable flora of rhizobia and nitrite forming organisms. They found that number of nitrite formers reached a peak in the spring, but that no such peak existed for rhizobia. Finally, in 1946, Appleman and Sears (10) made a study of nodulation on the Morrow plot soils, and again found more root nodule bacteria on the fertilized plots, especially where the appropriate legume was grown. They also concluded that the bacteria were spread readily in barnyard manure, but that wind action played very little part in disseminating the organisms.

From these papers, it would appear that rhizobia can survive very easily as free living organisms in the soil, under proper conditions, and that "natural" processes for spreading the organisms are usually very slow.

(2) Nodulation and Cross-Inoculation Groups.

Originally the root nodule bacteria were thought to constitute a single species, infecting all leguminous plants. Later workers found a certain specificity of action whereby certain strains of organisms appeared to infect some species of legumes but not others, and in 1932 Fred, et al (24) listed a number of "cross-inoculation groups", as determined by numerous earlier workers, in which the root nodule bacteria were divided into species on the basis of their power to form nodules on particular species of legumes, one species of rhizobia infecting a smaller or larger group of legume species.

However, it has become obvious that the problem is not so easily settled. Allen and Allen (3), Wilson, Burton and Bond (86), Kroulik and Gainey (43), Nutman (55), and Burton and Allen (16) all found more effective nodulation and, especially, nitrogen fixation by certain strains of a given species than by others. Newton and Wyatt (53) emphasized the need for the use of effective strains of Rh. leguminosarum in the inoculation of peas grown on grey wooded soils. In addition, Burton and Allen (16) found that if crimson clover (Trifolium incarnatum) was inoculated with a mixture of strains of Rhizobium trifolii, some effective and some ineffective, a large percentage of the nodules were from the ineffective strains. However, the initial inoculation

was shown to be the important one. Later inoculation, after nodules had been formed, showed no effect when either "good" or "poor" strains were used. Kroulik and Gainey (43) were interested in a possible correlation of resistance to infection by Rh. meliloti with resistance to Phytomonas insidiosa, the alfalfa wilt organism. They found no correlation between the two, but found a highly significant difference in the performance of different strains of Rh. meliloti with respect to infectivity, and found that strains of both plant and bacteria were important.

The work of Nutman (55) at Rothamsted is of particular interest. In a study of "effective" and "ineffective" strains of clover nodule bacteria he found no evidence of a change in "effectivity" due to plant passage, but found that storage in the soil (a Woburn sandy soil) seemed to cause the production of a large number of ineffective variants of effective strains. In view of the present popularity of humus cultures for legume inoculations, his results appear worthy of careful consideration. Similar variants occurred, but far less frequently, in effective strains stored as stock cultures on agar slants.

The appearance of strain variation complicated the theory of cross-inoculation groups, and further work indicated that the trouble was far from over. Carroll (17) found nodulation outside the cross-inoculation groups, and concluded, after a study of forty-one legume species from

fourteen genera, that "the present day conception of, and practices in, legume group inoculation are illogical and unsound and should be revised." Wilson, Burton and Bond (86) found that certain species and certain strains of legumes and bacteria gave erratic nitrogen fixation performance, but suggested that the carbohydrate-nitrogen relationship in the host plant was responsible. Appleman and Sears (8), however, found nodulation between cross-inoculation groups, and J. K. Wilson, (82, 83), cited five hundred instances in which a plant could be assigned to more than one cross-inoculation group. He suggests that from the scientific standpoint, the boundaries of the groups overlapped so much as to be useless for species differentiation. From a practical point of view, it would appear that, while Wilson's viewpoint may be somewhat extreme, attention to "effective" and "ineffective" strains is very important in the preparation of legume inoculation cultures.

(3) Legume Seed Inoculation

Once the existence and functions of the root nodule bacteria were recognized, it was soon realized that the bacteria were not native to all soils, and steps were taken to establish in soils originally deficient in the organisms, a population of the desired species of bacteria.

The soil transfer method, in which greater or smaller amounts of soil known to contain the desired organisms

were spread over the field to be inoculated, was introduced by Salfeld (as reviewed by Fred et al (24)). Because of the inconvenience and labor involved, this method was soon replaced by others in which the bacteria were applied directly to the seed. Of these early methods, the most successful consisted of simply dusting the seed with soil containing rhizobia. Various modifications were less successful.

Nobbe and Hiltner (as reviewed by Fred et al (24)) first introduced pure culture inoculants, in which the bacteria were grown on the surface of a jelly medium and applied either directly to the seed or to a small amount of soil.

The use of moist soil was introduced by Simon (as reviewed by Fred et al (24)). A pure culture of the desired species of rhizobia was grown in sterilized soil. Since that time the use of this type of culture has been extended until it is used almost exclusively. A number of workers made studies of the effectiveness of these cultures, and nearly all arrived at the general conclusion that the medium used was a suitable habitat for the rhizobia, and that it could be conveniently used to inoculate the seed. In addition, Albrecht (1) tested a dry soil culture, which he found to be unsatisfactory. Fellers (23) studied three types of commercial cultures, agar, liquid and soil and

muck cultures. He made plate counts of the numbers of bacteria and also made tests of the nodulation power of the cultures when applied to legumes. He gave a number of reasons for expecting humus cultures to replace agar or liquid cultures:

(1) Soil or muck cultures possess greater viability than agar or liquid cultures.

(2) More bacteria are usually present in acre size soil or muck cultures than in acre size agar or liquid cultures.

(3) In soil or muck there is better aeration and the medium is a more natural one than agar or liquid cultures.

(4) When humus or muck is used, many types of rhizobia may be added to the culture.

(5) Sterilization of soil or peat is not always necessary or desirable since some organisms present in soil aid the development of rhizobia.

(6) Heat, light and exposure do not affect soil and peat cultures as much as agar or liquid cultures. Even after soil or peat becomes dry, it will support the growth of many bacteria.

(7) The ease of inoculating with soil cultures gives them preference over liquid cultures.

Some of these points may be open to question, but in general their applicability is as great today as at the time of Fellers' work.

Some work was done on the minimum and optimum numbers of bacterial cells in inoculation cultures. Results were not entirely in agreement with each other, but Fellers (23) concluded that a minimum of 1,000,000 cells per ml. was necessary to secure inoculation, and Hedlin (31) used 1,000,000 cells per gram dry weight of humus culture as determined by the plate count method, as a limiting point in determining the effective life of humus cultures.

Some mention should be made of the desirability of legume inoculation, and of more recent developments in legume inoculation. Whiting (79) found that inoculation of canning peas with the proper strains of Rhizobium leguminosarum improved the yield and quality of the crop. Albrecht (1) obtained similar results with peanuts, but Vandecaveye (70) found that while inoculation, in Washington State, was satisfactory with the inoculants used, there were more or less undefined factors in the soil which adversely affected the subsequent growth and yield of alfalfa. Erdman (29) discusses a number of new developments in legume inoculation, including the advantages of using selected strains of rhizobia, and the complicating factor of chemical seed treatments, a bogey which had not arisen to plague the early investigators. He found that Spergon was the only chemical available which was not fatal to the bacteria used in inoculation, and the results obtained with Spergon were not conclusive. Baur (11)

obtained better results where chemical seed treatments were used, by bulking the inoculum with relatively large amounts of soil, muck or similar materials and sowing it in the drill row with the seed through the fertilizer attachment.

Kadow, Allison and Anderson (37) found that the surest method of securing good inoculation when chemical seed treatment was used, was to establish the proper bacteria in the soil. Of three seed treatments used - Semesan, cuprous oxide and Vasco 4 (zinc oxide) - only cuprous oxide failed to reduce nodulation when commercial seed inoculants were used. Apparently when the soil was fairly dry, cuprous oxide did not interfere with inoculation, but the data were too limited for this to be established conclusively. It was also found that the application of graphite to the inoculated seed prior to chemical disinfection appeared to exert some protective action, but this was not definitely established either.

Kernkamp (38), on the contrary, concluded that the use of Spergon as a disinfectant did not interfere with inoculation by "Nitragin" cultures. However, these results may be regarded with some suspicion, as the uninoculated controls grown in autoclaved soil developed rather large numbers of nodules.

With regard to the effectiveness of the cultures

now available, Collins (18) found all brands of commercial cultures available equally effective.

Three other lines of investigation should be mentioned. The idea of inoculation of seed prior to sale is not new, but Orcutt and Whitman (56) reopened the question with the statement that they found living root nodule bacteria on the seeds one year after inoculation, where chemical disinfectants were not used. However, there is considerable doubt as to the value of this method.

The use of mixtures of different species or strains of rhizobia in commercial cultures has become very common today. Hofer (34) and Bond (13) found that the mixed cultures were as effective in nodulation as single strain cultures.

Appleman and Sears (9) suggested an entirely new method of inoculation by using lyophiled (frozen and dried under vacuum) cultures as inoculants. In their experiments, the lyophiled bacteria were mixed with talc, calcium carbonate, or aluminium hydroxide to give bulk. The organisms survived up to thirteen weeks after mixing with the carrier, either on the seed or in test tubes.

Moodie (49) made a summary of benefits from inoculation, reasons for poor results, reasons for inoculating every seeding, selection of the proper culture, and a list of the brands of inoculants available. Thornton (68) emphasized

the need for inoculation with experiments showing the slow rate of migration of rhizobia through soil. Demolon and Dunez (21) confirmed this finding with a study of the spread of soy bean bacteria in experimental plots.

(4) Growth and Survival of Root Nodule Bacteria

Fred et al (24) listed nine factors other than energy source influencing the growth of rhizobia: air supply, temperature, drying, sunlight, reaction, presence or absence of various inorganic substances, vitamin B, dyes, and antagonisms and symbiosis. The effect of dyes is of little importance in the survival of rhizobia in soils, and the effect of light of very little more. Nobles (54) was the only worker reviewed by Fred et al (24) to find a noticeably harmful effect of light on root nodule organisms in soil. It is undoubtedly true that light, especially of certain wave lengths may be fatal to the unprotected bacteria.

However, Fred et al (24) made no mention of another factor, which has become more prominent of recent years, the effect of bacteriophage. This factor has been encountered in widely separate regions, and appears to be of sufficient importance to be discussed separately. The other factors listed above may be discussed in the order given previously.

(a) Air Supply

Beijerinck, as reviewed by Fred et al, (24)

mentioned (1888) the apparent need for oxygen in the growth of root nodule bacteria. Burris and Wilson (14) found a maximum rate of respiration at partial pressures of oxygen of 0.1 to 0.15 atmospheres. Konishi et al (42), however, found a repression of respiration of Rh. trifolii at oxygen tensions below that of normal air.

Little and Burris (46) found the respiration of root nodule bacteria at low oxygen tensions to be stimulated by the presence of horse hemoglobin and to a lesser extent by the related red pigment found in root nodules. Hedlin (31) found that humus cultures of rhizobia died out rapidly when stored in sealed glass containers. Many other workers, as reviewed by Fred, et al (24), have obtained similar results, but on the other hand Jones (36) stored cultures of rhizobia for 11 to 15 years in small Freudenrich flasks containing a modified Ashby's agar. The flasks were plugged with cotton, sealed with wax and stored at room temperature. The same medium was used when the organisms were plated out, and all 12 cultures used produced typical Rhizobium colonies.

The oxidation-reduction character of the medium is also important, as was shown by Allyn and Baldwin (7). They demonstrated by means of agar shake and petri dish cultures that the best growth of rhizobia occurs when the medium is poised at the proper level.

(b) Effect of Temperature

Beijerinck, as reviewed by Fred et al (24) gave

the thermal growth range of rhizobia as 0° to 47°C, and later workers, also reviewed by Fred et al, (24), have confirmed the correctness of this very closely as 0° to 50°C, with an optimum between 20 and 28°C. Ockerblad was mentioned as having reported the thermal death point of rhizobia in liquid media as between 59° and 61°C with 10 minutes exposure. However, Gangulee (29) observed that in the soil alfalfa bacteria remained alive after exposure to 50°C. for seven days.

Later workers such as Wilson (84) found a growth optimum at 28° to 30°C. There was a decrease in rate of respiration at temperatures approaching 40°C. Allison and Minor (6) obtained similar results. Koffler, Johnson and Wilson (41) found that increased temperatures brought about an irreversible denaturation of respiratory enzymes, in contrast to the reversible denaturation brought about by urethane at suboptimal temperatures. Rhizobia, however, were found to be very resistant to low temperatures. Breal, Fred and Frazier, as reviewed by Fred et al, (24), found no evidence that freezing of the substrate caused injury to the rhizobia. Vass (75) froze Rhizobium leguminosarum cultures in a mannitol liquid, sand, and soil at temperatures ranging from -15°C to -190°C for 3 minutes to 6 hours. All cultures were found to be alive at the end of the experiment. There was a gradual decrease in the numbers of bacteria in

the mannitol solution as a result of the longer freezing periods, but not in sand or soil, even at -190°C.

(c) Effect of Drying:

The sensitivity of rhizobia to drying was found to depend largely on other environmental factors. Harding and Prucha, as reviewed by Fred et al (24) found that rhizobia cannot survive on wads of cotton under ordinary atmospheric conditions. Chester, also reviewed by Fred et al (24), in 1907, found that in thin films on the surface of glass, they die rapidly. Temple (62), Vandecaveye (70), and Giltner and Langworthy (30), all found that rhizobia remained viable in air-dry soil for a remarkably long time. The bacteria were found to be much more resistant to drying in a clay loam than in sand. It has been found more recently that lyophiled (quick frozen, dried and stored under vacuum) cultures of rhizobia may survive a good many years. However, this involves factors not occurring in the study of survival of rhizobia in soils.

(d) Effect of Reaction

The reaction of the medium was early found to be worthy of study in connection with the growth of Rhizobium species. Moore, Maassen and Muller, Mazé, Zipfel, Richmond, Salter, and Fred and Loomis were reviewed by Fred et al (24), and were of rather different opinions as to the effect of

reaction on rhizobia. Moore, in 1905, stated that the nodule bacteria would stand any degree of acidity which would permit the growth of the host plant. Maassen and Muller considered the bacteria very sensitive to the reaction of the medium.

Maze divided the nodule bacteria into those accustomed to acid soil and those accustomed to alkaline soils. Zipfel observed that small amounts of acid or alkali did not affect the growth of the bacteria he studied. Richmond, in 1926, reported that cowpea and soybean bacteria were killed in acid soil within 3 1/2 years.

Prucha, using alfalfa bacteria on agar slants, showed the bacteria to be more sensitive to HCl than to equivalent amounts of NaOH.

Salter, and Fred and Loomis, found that a reaction near neutral was best for the growth of alfalfa and clover bacteria. Fred and Davenport (25) found that bacteria from different cross-inoculation groups behaved differently towards acidity. The lower critical pH for alfalfa bacteria was found to be 4.9, for lupine, 3.2. Thornton (67) found that a pH of 6.0 or higher was necessary for the survival of Rh. trifolii in some Florida soils.

Where nodulation was concerned, Fred, Whiting and Hastings(27) found that the limits for nodule production in solution cultures were about pH 4.6 to 8.0, and for growth, about pH 3.9 to 9.6. The most favourable reaction for growth and nodulation was about pH 6.5. For alsike clover, growth

limits were between pH 5.0 and 10.0, with maximum growth and nodulation between pH 6 and 8.

(e) Effect of Inorganic Substances:

The elements listed by Fred et al (24) required by rhizobia for growth are those known to be required by higher plants. Phosphorus and potassium were reported as being essential for growth by several workers, including Truesdell (69), Bewley and Hutchinson (12) and Lohnis (48). Nitrogen compounds, while preventing nodulation when present in fairly high concentrations (Wilson (85)) are necessary for the growth of root nodule bacteria in absence of the host plant. Hills (32) found that 250 p.p.m. nitrate nitrogen in soil cultures of Rh. meliloti stimulated growth of the bacteria. Higher concentrations had a depressing effect, and concentrations over 1,000 p.p.m. were found to be toxic.

Calcium was found to be beneficial by a great many workers. Fulmer (28) found a stimulating effect by calcium carbonate on the growth of rhizobia in Colby silt loam, but this may have been because of the production of a more favourable soil reaction. Alicante (4) obtained similar results. Pitz, as reviewed by Fred et al (24) observed a beneficial effect on the multiplication of rhizobia accompanying the addition of calcium sulphate to the medium.

Later studies have been concerned with attempts to determine the effects of trace elements and other factors on the growth of rhizobia. Albrecht and McCalla (2) found that calcium absorbed on colloidal clay supplied with other nutrients maintained normal growth of rhizobia. Steinberg (61), in the study of trace element requirements of rhizobia found that thorough purification of the chemicals used in the nutrient solutions gave slightly more clear-cut results at low nutrient levels. Lilly and Leonian (45) reported on the interaction of iron with several accessory growth factors in the growth of Rh. trifolii. They used thiamine, pantothenic acid, inositol, pyridoxine, nicotinic acid, riboflavin and p-amino benzoic acid alone and in various combinations, with and without iron. All were found to be stimulatory to growth under specified conditions. Mulder (50) stated that molybdenum was apparently required in symbiotic nitrogen fixation.

(f) Vitamin B and Other Accessory Factors:

Fred et al (24) merely mentioned that a large number of microbiologists had investigated the relation of vitamins to the growth of microorganisms. However, Allison and Hoover (5), Wilson, (85), Lilly and Leonian (45), and other recent workers have made it clear that many of the members of the B complex are accessory factors in the growth of rhizobia.

(g) Antagonisms and Symbiosis:

The effects of the presence of other micro-organisms in cultures of rhizobia attracted the attention of a good many of the earlier workers. Fred et al (24) reviewed twenty papers dealing with the effects of other microorganisms on the growth of nodule bacteria. Of these, six cited beneficial effects as a result of the association, though the results were somewhat contradictory. Hino (33) was the only worker to report rhizobia as not being eaten by protozoa.

Robison (57, 58) isolated six antagonists of legume bacteria. These were all common soil microorganisms, including Aspergillis wentii, which produced a bacteriostatic agent reducing the numbers of nodules on Trifolium repens. Furthermore, Hedlin (31) found that competition by other microorganisms greatly reduced the numbers of rhizobia in humus cultures. A paper by Lochhead and Landerkin (47) is of interest. In studying the antagonisms existing between Streptomyces scabies and other actinomycetes, they found that cross-antagonisms between the various antagonistic organisms were very numerous. This suggests that normally a particular organism antagonistic to any other would be unlikely to multiply to such an extent that the growth of the second organism would be completely suppressed. Thus, because of the complex nature of the microstructure of the soil, it is

normally unlikely that the population of rhizobia in soil would be unduly reduced by the action of other microorganisms.

(5) Bacteriophage:

The discovery of the influence of bacteriophage is comparatively recent in the study of legume inoculation, and the study is by no means complete, but its potential importance cannot be ignored. Demolon and Dunez (20, 21), in France, made a study of "fatigue" of soils growing legumes continuously for several years. They found reduction in yield to be due to decreased nodulation and, hence, nitrogen fixation. This was caused by a relatively non-specific bacteriophage attacking all species of rhizobia. The effects of the bacteriophage as shown by yield and nitrogen content of the crop could not be entirely offset by the use of nitrogenous fertilizers. They advocated as a remedy inoculation with phage resistant strains of bacteria.

Vandecaveye and Katznelson (73) in Washington isolated a lytic principle effective against 80 per cent of their stock cultures of Rh. meliloti. They failed to find the phage in alfalfa fields less than three years old. However, fields three or four years old were a good source of the phage. It was felt that poor alfalfa yields were frequently due to the presence of phage. Vandecaveye, Fuller, and Katznetson (72) found the same decreases in yield in alfalfa

grown two or three years on the same land. It was found that application of nitrogenous fertilizers increased yields markedly, but that inoculation with phage-resistant strains had no effect. The reduction in yield was as high as 60 - 70 per cent, and it was shown that disease of the plant was not the cause. Vandecaveye and Moodie (74) later found that the use of phage-resistant strains of bacteria, or of different strains of alfalfa, had no effect. Again, the use of nitrogenous fertilizers increased the yield.

Kleczkowska (39, 40) at Rothamsted, developed an agar plate method for counting phage particles, and made a study of the interaction of the bacteriophage with rhizobia. The results were extremely interesting, especially from a practical standpoint.

(1) The phage attacked only 10 - 15 per cent of the pea and clover bacteria strains tested. These strains were collected over a large area.

(2) The phage was not unusual in its specificity.

(3) Single small samples of turf contained both resistant and susceptible strains of rhizobia.

(4) In the soil, a rapid development of resistant bacterial strains took place.

(5) When a lysed culture had cleared, in a few days a further growth of the bacteria occurred. These organisms were found to be susceptible to the phage, but were protected

from lysis by some principle produced in the culture medium.

(6) Resistant dissociants were as effective in nitrogen fixation as the parent strain.

(7) Aseptically grown clover, inoculated with susceptible bacteria, produced resistant variants in the absence of the phage.

These results differ markedly from those obtained by Vandecaveye and his coworkers, and from those of Demolon and Dunez (20, 21), in that they suggest that neither phage-resistant strains, as suggested by Demolon and Dunez, or nitrogen fertilizer, to which Vandecaveye finally resorted, are necessary. It may be possible that the different environmental conditions prevailing in the three different countries had some effect.

(6) Respiration Studies of Soils and Rhizobia:

Measurements of respiration, by various means, have been used for some time in studies of the metabolism of the different Rhizobium species. Walker, Anderson and Brown (76, 77) carried out experiments to determine the effect of nitrogen source on four species of rhizobia. At that time the need by rhizobia for various growth factors was little understood, and the lack of these growth factors, while recognized by Walker and his coworkers, exerted a masking influence on the results of the work.

Thorne and Walker (65) and Neal and Walker (51, 52) carried out extensive experiments using measurements of the oxygen uptake to determine the effect on the growth of rhizobia of reaction of the medium, and to study the utilization and the extent of oxidation of carbonaceous materials. Thorne and Walker (66) later made a study of the effect of accessory growth factors on the oxygen uptake and carbon dioxide production of rhizobia. At that time the pure growth factors were unknown and various plant, soil and bacterial extracts were used as sources of growth factors.

Konishi et al (42) measured the respiration of Rhizobium trifolii, Rhizobium lupini, and Rhizobium meliloti under different pressures of oxygen and carbon dioxide. They found respiration to be reduced when the oxygen tension was below that of normal air, though the respiration of Rhizobium trifolii was depressed the least. The respiration of Rh. trifolii was found to be promoted in a partial pressure of carbon dioxide of 0.05 atmospheres. These results were not entirely in agreement with those of Burris and Wilson (14) who found the maximum rate of respiration to be reached at a pO_2 of 0.1 to 0.15 atmospheres.

Wilson (84) developed a technique for using the "resting cell" technique for studying the metabolism of rhizobia, and found a growth optimum at 28° - 30°C and a respiration optimum at 37°C. The respiration rate decreased with time near 40°C. Thorne and Burris (64) used this

technique and a technique for making suspensions of rhizobia directly from the nodule to compare the respiration of root nodule bacteria from nodules and from stock laboratory cultures. There was some evidence that the cells from the nodules reached a maximum rate of respiration at a lower oxygen tension than did bacteria from stock cultures. Hoover and Allison (35), however, made respiration measurements on aliquots taken directly from growing cultures, avoiding the washing necessary to obtain "resting" cells. They also made a complete accounting of materials used and products formed. Both methods have their advantages.

Burris et al (15) used the Warburg respirometer to compare the adaptation mechanisms of Rhizobium and Azotobacter, Little and Burris (46) in 1947, used the same type of apparatus to show that respiration of nodule bacteria at low oxygen tensions was stimulated by hemoglobin and by the very similar red pigment of root nodules, and in the same year Koffler, Johnson and Wilson (41) determined the combined influence of temperature and urethane on respiration of rhizobia.

The measurement of the respiration of microorganisms in the soil is a different problem from that of making the same sort of determination on a pure culture of bacteria. Usually a much larger sample of soil is necessary, several grams of sample at least instead of a few milligrams, for

uniformity. In addition, the interactions between the bacteria and the enormous surface area present in soil are almost impossible to determine, and the micro-structure of the most uniform soil sample is extremely heterogeneous.

Relatively little work has been done on soil respiration. Smith and Brown (60) in 1932, used a modified Barcroft differential manometer to show that carbon dioxide production of Iowa soils followed the bacterial growth curve. Corbett (19) followed the evolution of carbon dioxide from tropical soils by an absorption method, and found results similar to those of Smith and Brown while the rate of carbon dioxide production was increasing. Finally, Lees (44) described a modification of the Haldane constant-pressure respirometer, which offered a method of measuring the respiration of pure cultures of rhizobia, in soil as a substrate, and this method was used in the series of experiments described here.

MATERIALS AND METHODS

The work reported here was divided into a study of the growth and survival of root nodule bacteria in mixed humus and soil, and in soil cultures under various treatments, and a study of the oxygen uptake of Rh. meliloti in six soils from two Southern Alberta irrigation districts. Straw and alfalfa had been allowed to decompose for periods of approximately two to eight weeks in samples of these soils before they were steam sterilized and inoculated with Rh. meliloti.

(1) Growth and Survival Experiments:

Inoculant bases used were:

(1) A mixture of equal portions by weight of black mineral soil from the Soils Department plots south of the city of Edmonton with one of two peats obtained from near Winterburn and about four miles north of Breton.

(2) A peat-carbon black mixture obtained from Mr. J. A. Robertson of Edmonton, who used it as a base for commercial legume inoculants.

(3) A brown soil obtained near Lethbridge, which was used in one experiment to study the effect

Materials and Methods

The work described here was divided into a study
of pH stability and activity of water soluble products in
which there was still, due to small difference under certain
circumstances, the study of the oxygen content of Bi.
<u>megaterium</u> in more cells than the secondary effects introduction
elsewhere. There are slightly two methods to determine
the amounts of concentrations by the right means to examine
of cells with which there were already classified and
investigated with Bi. <u>megaterium</u>.

(1) <u>Oxygen and Material Measurements</u>

Investigations were made:

(A) A reduction of total content by method of disk
filtered cell free bio fluid concentrated plate
study on the ability of immersion with one of two
cells obtained from deep fermentations and about
four cells ounce of bacteria.

(B) A test-tubes flask methods obtained from Bi.
T. A. Foord-Penn of Hamilton, who used it as a
base for commercial Degree Investigate.

(C) A Foord cell chamber deep immobility, which
was used in one experiment to study the effect

of phosphate on the survival of rhizobia in soil.

In experiments designed to study the effect of different nutrient and other treatments on the growth and survival of rhizobia in the different inoculant bases, the cultures were stored in Erlenmeyer flasks of appropriate sizes and loosely stoppered according to the method used by Hedlin (31). The treatments used were $CaCO3$, $MgCO_3$, $K2HPO_4$, $CaSO_4$, sucrose, mineral oil, $NaNO_3$, and sodium alginate, alone and in various combinations and concentrations. Six experiments were set up in which the suitability of two types of screw cap jars as containers was investigated. One type was a narrow-necked brown jar, in which the jar was plugged with cotton before the cap was put on, and the other was a wider mouthed 8-oz. clear glass jar from which the cotton plug was omitted. Cultures stored in jars were given similar treatments to those received by cultures in Erlenmeyer flasks. In all cases, the inoculant bases were given the appropriate treatments, made up to forty per cent of total water holding capacity, sterilized for the length of time necessary for the soil used, according to the figures obtained by Simpson (59), and inoculated with 1.0 cc of a suspension of the bacteria grown in a yeast-extract sucrose solution, as recommended by Fred and Waksman (26). In

general, the initial number of bacteria per gram of base was relatively low.

In all cases, counts of the numbers of bacteria were made by the plate method, using the yeast extract sucrose agar containing congo red, recommended by Fred et al (24), and by Fred and Waksman (26).

Pure cultures were used throughout the experiment, as Hedlin (31) had found that the presence of contaminating organisms reduced the numbers of root nodule organisms considerably, and it was found that contamination of the stored cultures was relatively rare.

(2) Oxygen Uptake by Soil Cultures of Rh. Meliloti:

Six soils from south-western Alberta were used as media in which the bacteria were grown, five of these soils being from the Magrath Irrigation District, and one from the east end of the Mountain View Irrigation District, one mile west of Cardston. The Magrath soils were from the dark brown soil zone, and the general area is classed as clay and silty clay loam, though there are patches of lighter soil in the district. The Cardston soil was from the shallow black soil zone, and is a loam or sandy loam. These soils were chosen because the Cardston soil has been irrigated for only two or three years, while the Magrath Irrigation District is the oldest large project in Alberta, having been

irrigated for fifty years at the time the samples were taken. It was felt that if there were adverse effects from long continued irrigation, it would be more easily detected in these soils. Five of the soil samples were taken from fields growing alfalfa at the time, but soil number 3 was a sandy soil (loam) from a sugar beet field known to have been irrigated heavily for all of the fifty years of the operation of the district.

The soils were as follows:

(1) C. C. Spencer, Magrath, Silty clay loam, low lying, tendency to alkali. pH 7.5.

(2) N. L. Hull, Cardston. Shallow black soil from Cardston, silt loam, high in organic matter, newly under irrigation. pH 6.3.

(3) K. Hindly, Magrath. Loam, heavily irrigated for fifty years. (In sugar beets - 1950). pH 8.0.

(4) O. Owens, Magrath. Clay, lightly irrigated. pH 7.6.

(5) W. Dudley, Magrath. Silty clay beside fertilizer trials. Excellent hay crop in 1950. pH 8.1.

(6) J. A. Spencer, Magrath. Silty clay loam, beside fertilizer trials. Also good hay crop in 1950. pH 7.4.

Two samples of each soil were placed in tumblers and 2 per cent by weight of chopped straw and alfalfa respectively were

added to the samples, which were then brought up to 50 per cent of the total water holding capacity of the soil. The moisture content was maintained by adding water at intervals and samples were taken every ten days to two weeks, dried at 45°C and stored in 4-oz. jars.

The samples thus taken were ground in a mortar, and duplicate samples of twenty grams were weighed into the reaction vessels of a modified Haldane respirometer, as described by Lees (44). The procedure and apparatus used by Lees were further modified by substituting a 25 ml. Mohr burette for the 5 ml. pipette making a correction to the burette reading for changes in room temperature, and replacing the air in the reaction vessels with pure oxygen. These modifications enabled the oxygen uptake to be followed for up to three days without opening the reaction vessels.

After adding the soils to the reaction vessels, they were moistened with sufficient water to bring the water content up to 50 per cent of total water holding capacity, less 5 ml. which was later added as inoculum, plus 2 ml. to allow for loss in the autoclave. The soils were then stirred to break up the lump of puddled material formed when the water was added, the absorption cups and filter papers were placed in the reaction vessels and the vessels were stoppered with cotton and sterilized.

The inoculum was prepared by the method of Wilson (84) except that facilities were not available for concentrating and washing the bacterial cells by centrifugation. Two Roux bottles, each containing about 100 ml. of carbohydrate free media containing 0.1 per cent yeast extract, the salts of medium 79 (Fred and Waksman (26)) and 2.5 per cent agar were inoculated with 3 ml. of a suspension of Rh. meliloti obtained by washing a 48 hour culture of the organisms from a slant of the same medium. The Roux bottles were incubated for 48 hours, and the bacteria washed off the agar with physiological saline solution into a bottle containing glass beads. The suspension thus obtained was then filtered through sterile glass wool to remove clumps, and was relatively free from gum. The soils were inoculated with 5 ml. of the suspension thus obtained, oxygen was passed through the reaction vessels for fifteen minutes, the absorption cups were filled with 10 per cent NaOH solution, and the vessels were placed in a water bath at 30°C. and attached to the respiration apparatus. After being allowed to remain open to the air at least fifteen minutes to reach thermal equilibrium, the pinch clamps were closed, and the first set of readings of the burette were taken. Readings were then taken every two or three hours during the day over a period of two days. In all cases, a "blank" determination in the untreated soil was included. The number of mls. of O_2 absorbed was calculated and converted to standard temperature and pressure.

(3) Field Fertilizer Trials With Minor Elements:

Three fertilizer trials were placed on alfalfa in the Brooks district, and two in the Magrath district, to test the effect of minor elements on the yield of alfalfa, but the results were in all cases negative.

(4) Counts of Rhizobium in Field Soils:

Counts of Rh. meliloti were made on four soils from the vicinity of Edmonton, by the method of J. K. Wilson (80). Since nodulation on these fields had been very poor for some time, and the presence of root nodule bacteria was uncertain, it was deemed advisable to make an attempt to make some estimate of the numbers of rhizobia present.

RESULTS

(1) Plate Counts of Rhizobia in Humus and Soil Cultures

(a) Results of Experiments with Inorganic Nutrients and Sucrose.

The results of these experiments are presented in Tables I - VII. In Tables I and II are the results of experiments designed to follow the growth of rhizobia over the first month after inoculation of the humus base, although the experiment reported in Table II was later extended to 298 days. In both experiments the same nutrient treatments were used, and plate counts were made every three or four days. In the peat-black soil mixture (Table I) all treatments gave higher plate counts than did the check, except where magnesium carbonate alone was added, in which case the plate counts for the sixteenth and nineteenth days fell below that of the check. Where sucrose was present, the numbers rose less rapidly, but by the twelfth day, the counts of cultures to which sucrose had been added had risen to over 2500 millions per gram, and continued to rise to 3450 millions per gram on the sixteenth day. The counts of all other cultures had reached a maximum about the ninth to twelfth day, and decreased to about 200 - 400 millions per gram on the 32nd day. On the 32nd day the count of the

culture to which sucrose had been added was 840 millions per gram. By this time the average water loss from the cultures had reached 4.8 grams.

The experimental results in Table II, in which peat-carbon black mixture was used as a base, were rather different. In this base the difference between the counts of rhizobia in the check and those of the treated cultures was much less, except where sucrose was added to the medium. In the cultures containing sucrose the counts had risen to 5150 millions per gram by the fifth day, and at the end of 30 days were still over 4,000 millions per gram, except for three counts which dropped below that level. Counts of all other treatments reached a maximum between about 3000 and 4500 millions per gram, which was reached somewhat later in those cultures to which dipotassium phosphate had been added although the results were somewhat irregular. These results were not obtained in the peat-black soil mixture.

This experiment was continued for 298 days, at the end of which time the highest count was 6.5 millions per gram, in the cultures to which calcium carbonate alone, and all nutrients plus sucrose, had been added. The count on the untreated base was 5.7 millions per gram.

The average water loss was 3.8 gm. after 30 days, and 31.7 gm. after 298 days.

TABLE I. Plate counts of Rh. leguminosarum (K_1) in peat-black soil mixture given various treatments, sterilized, inoculated and incubated in erlenmeyer flasks stoppered with cotton, at room temperature. (millions per gram.)

Incubation time in days	No nutrients added	$MgCO_3$ (0.5%)	$CaCO_3$ (0.6%)	$CaCO_3$ (0.6%) $MgCO_3$ (0.5%) K_2HPO_4 (0.1%)	$CaCO_3$ (0.6%) $MgCO_3$ (0.5%) K_2HPO_4 (0.1%) $CaSO_4$ (0.2%)	$CaCO_3$ (0.6%) $MgCO_3$ (0.5%) K_2HPO_4 (0.1%) $CaSO_4$ (0.2%) Sucrose (2.0%)	Average Water Loss in gm. *
0	1.0	1.0	1.0	1.0	1.0	1.0	0.0
3	64.0	472.0	931.0	1833.0	1621.0	161.0	0.4
6	319.0	7.4	1208.0	1071.0	1326.0	862.0	0.4
9	401.0	671.3	949.0	962.0	1021.0	977.0	1.0
12	0.0	228.0	621.0	652.0	727.0	2599.0	1.6
16	576.0	427.0	830.0	772.0	830.0	3450.0	1.6
19	494.0	442.0	630.0	890.0	950.0	1930.0	2.2
21	306.0	425.0	650.0	670.0	770.0	1560.0	2.8
24	0.0	0.0	107.0	492.0	560.0	708.0	3.0
27	219.0	296.0	360.0	575.0	372.0	680.0	3.8
32	163.0	210.0	330.0	320.0	339.0	840.0	4.8

* Total water added = 51 ml.

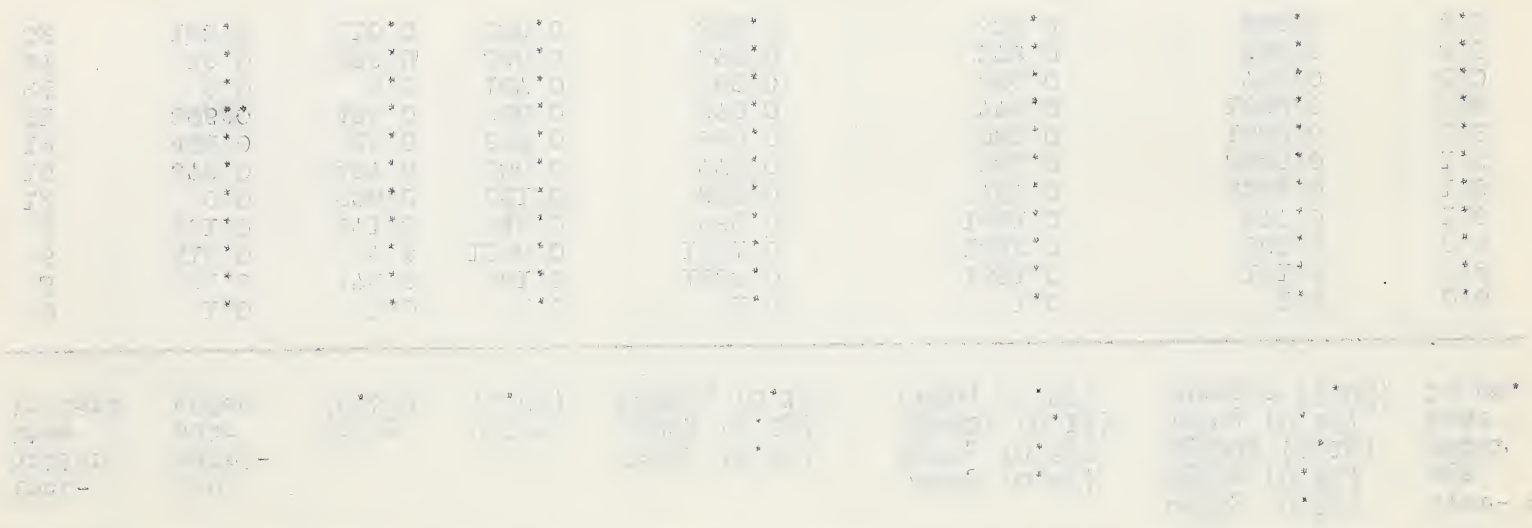


TABLE II. Plate counts of Rh. leguminosarum (K_1) in peat-carbon black mixture given various treatments, sterilized, inoculated and incubated in erlenmeyer flasks stoppered with cotton, at room temperature (millions per gram).

Incubation time in days	No nutrients added	$MgCO_3$ (0.5%)	$CaCO_3$ (0.6%)	$CaCO_3$ (0.6%) $MgCO_3$ (0.5%) K_2PO_4 (0.1%)	$CaCO_3$ (0.6%) $MgCO_3$ (0.5%) K_2HPO_4 (0.1%) $CaSO_4$ (0.2%)	$CaCO_3$ (0.6%) $MgCO_3$ (0.5%) K_2HPO_4 (0.1%) $CaSO_4$ (0.2%) Sucrose (2.0%)	Average Water Loss in gm. *
0	0.23	0.23	0.23	0.23	0.23	0.23	0.0
2	303.0	159.0	703.0	361.0	294.0	401.0	0.5
5	1922.0	2052.0	1570.0	3780.0	3530.0	5150.0	0.9
7	2462.0	2193.0	2690.0	2770.0	2370.0	5250.0	0.8
10	4662.5	2980.0	3010.0	3520.0	2800.0	3910.0	1.2
13	4110.0	2890.0	3400.0	4550.0	3380.0	5110.0	1.5
17	3780.0	1850.0	3030.0	2280.0	3580.0	4950.0	1.9
20	1710.0	1800.0	1165.0	1237.5	3560.0	3790.0	1.9
23	980.0	1280.0	1600.0	2630.0	4700.0	3060.0	2.3
26	3410.0	1040.0	2350.0	2150.0	3630.0	4000.0	2.7
30	1870.0	1880.0	1410.0	2040.0	2940.0	4210.0	3.8
97	396.0	3089.0	1210.0	534.0	830.0	960.0	13.0
137	14.0	53.0	166.0	104.0	0.0	0.0	14.8
228	22.6	53.4	35.0	239.0	22.4	423.0	27.7
298	5.7	1.6	6.5	2.1	3.8	6.5	31.7

* Total water added = 46 ml.

The results of adding dipotassium phosphate alone to the peat-carbon mixture are shown in Table III. The counts obtained were somewhat erratic. However, when 0.20 and 0.40 per cent K_2HPO_4 was added, there was some indication that a higher count was obtained. In this experiment the highest count obtained was 3790 millions per gram, as compared to 5250 millions per gram where sucrose and potassium phosphate were added to the same medium, and the average water loss after 252 days was 36.2 gm.

Table IV gives the results of plate counts of rhizobia in peat-black soil mixture, given similar treatments to those used previously, and stored in 16-oz. brown screw top jars. The plate counts were much lower than those obtained in this medium before, but the peat was more coarsely ground when the medium was made up, and this may have affected the results. There appeared to be some stimulatory effect due to the addition of sucrose, but there was apparently little benefit derived from the other nutrients added. It is of interest to note that in 268 days the average water loss was only 13.2 gm.

The plate counts given in Table V were obtained from cultures grown in peat-carbon mixture in clear glass screw-top jars, half of which were stored in the refrigerator. The medium used was from a second lot of peat-carbon black mixture, obtained from discarded unused cans of commercial

TABLE III. Plate counts of Rh. leguminosarum (K_1) in peat-carbon black mixture given treatments of K_2HPO_4, sterilized, inoculated and incubated in erlenmeyer flasks stoppered with cotton, at room temperature (millions per gram).

Incubation time in days	No nutrients added	0.05% K_2HPO_4	0.1% K_2HPO_4	0.20% K_2HPO_4	0.40% K_2HPO_4	Average water loss in gm. *
0	1.4	1.4	1.4	1.4	1.4	0.0
4	3080.0	2470.0	1650.0	3100.0	2410.0	0.8
12	2710.0	2790.0	2060.0	3790.0	3180.0	1.9
25	2050.0	1040.0	2510.0	1700.0	2400.0	4.8
45	1180.0	1320.0	1720.0	1600.0	1570.0	6.8
131	101.0	175.0	161.0	204.0	210.0	20.4
181	26.0	56.0	42.0	119.0	150.0	26.5
252	0.0	0.0	0.1	0.0	0.2	36.2

* Total water added = 46 ml.

TABLE IV. Plate counts of Rh. leguminosarum (K_1) in peat-black soil mixture, given various treatments, sterilized, inoculated and stored in 16-oz. brown screw-top glass jars at room temperature (millions per gram).

Incubation time in days	No nutrients added	$CaCO_3$ (0.6%)	$CaCO_3$ (0.6%) K_2HPO_4 (0.2%)	$CaCO_3$ (0.6%) K_2HPO_4 (0.2%) Sucrose (2.0%)	Average * water loss in gm.
0	5.3	5.3	5.3	5.3	0.0
5	33.2	160.0	240.0	68.0	0.1
12	18.0	261.0	163.0	230.0	0.3
27	182.0	183.0	151.0	311.0	0.7
63	278.0	175.0	148.0	370.0	2.1
105	224.0	308.0	403.0	386.5	3.3
167	357.0	138.0	97.0	351.0	6.3
268	44.9	36.1	50.1	56.1	13.2

* Total water added = 54 ml.

TABLE V. Plate counts of Rh. leguminosarum (B_3) in peat-carbon black mixture, given various treatments, sterilized, inoculated and incubated in clear glass screw-capped 8-ounce jars, half at 0 - 3°C. and half at room temperature (millions per gram).

	Refrigerated					Room Temperature				
Incubation time in days	No nutrients added	$CaCO_3$ (0.6%)	$CaCO_3$(0.6%) K_2HPO_4(0.2%)	$CaCO_3$(0.6%) K_2HPO_4(0.2%) Sucrose(2.0%)	Average water loss in gm.*	No nutrients added	$CaCO_3$ (0.6%)	$CaCO_3$(0.6%) K_2HPO_4(0.2%)	$CaCO_3$(0.6%) K_2HPO_4(0.2%) Sucrose(2.0%)	Average water loss in gm.*
0	20.0	20.0	20.0	20.0	0.0	20.0	20.0	20.0	20.0	0.0
5	33.8	82.7	27.5	32.4	0.2	5206.0	6288.0	4938.0	2685.0	0.6
35	70.0	25.0	15.0	15.0	1.2	1460.0	1750.0	771.0	1660.0	4.2
93	125.0	40.0	40.0	40.0	2.7	564.0	722.0	536.0	196.5	14.2
175	1988.0	901.0	1260.0	37.6	3.5	237.5	509.0	191.0	644.0	20.7
265	1401.0	992.0	1198.0	1202.0	6.9	42.6	230.0	110.6	92.0	27.1
342	440.0	752.5	475.0	214.0	10.0	23.6	15.6	26.9	165.0	32.4
402	510.0	545.0	420.0	730.0	11.3	5.8	15.2	44.3	10.5	40.9

* Total water added = 50 ml.

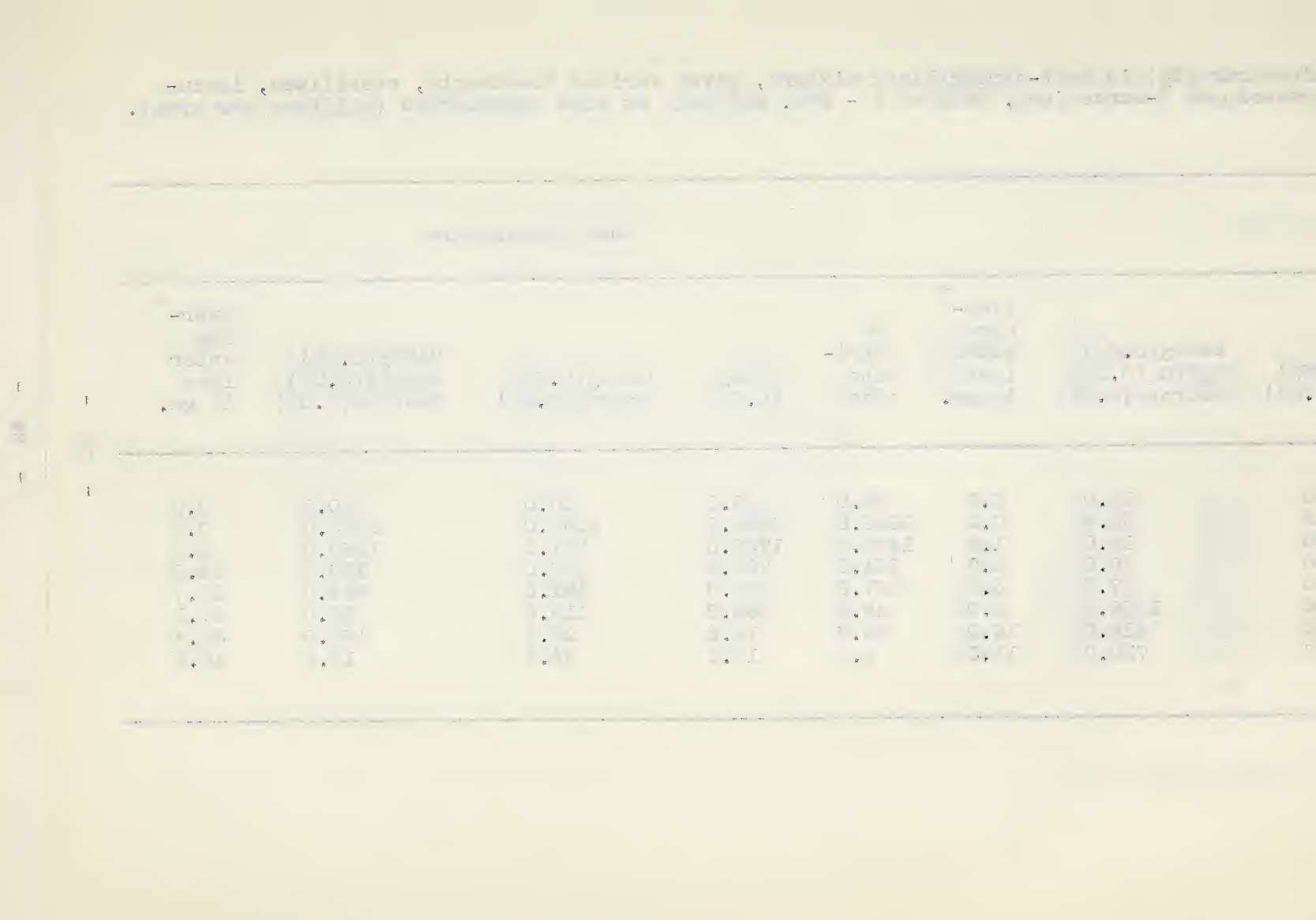


TABLE VI. Plate counts of Rh. leguminosarum (K_1) in peat-black soil mixture, given various treatments, sterilized, inoculated and incubated in erlenmeyer flasks stoppered with cotton, at about 7°C. and at room temperature (millions per gram).

Total incubation time in days	Incubation time in days at room temperature	No nutrients added	$CaCO_3$(0.6%)	$CaCO_3$(0.6%) + K_2HPO_4(0.2%)	$CaCO_3$(0.6%) + K_2HPO_4(0.2%) + Sucrose (2.0%)	Average water loss in gm. ≠
0	-	4.5	4.5	4.5	4.5	0.0
5	-	0.1	5.2	0.4	0.1	0.2
11	-	3.8	14.7	17.2	8.1	0.3
19	-	11.1	28.9	52.9	0.2	0.5
25	-	11.9	21.3	35.8	6.2	0.6
33	-	112.2	140.0	165.0	64.0	0.7
39	-	120.0	172.0	135.0	77.0	0.8
46	-	178.0	179.0	167.0	213.0	1.1
* 54	-	176.0	250.0	221.0	398.0	1.4
64	10	199.0	222.0	222.0	139.0	3.3
74	20	201.0	298.0	242.0	375.0	3.7
81	27	318.5	408.0	297.0	684.0	4.7
88	34	245.0	280.0	249.0	321.0	4.9
95	41	342.0	273.0	256.0	397.0	4.8
102	48	230.0	263.0	247.0	303.0	4.9
109	55	212.0	228.0	219.0	303.0	5.8
119	65	121.0	146.0	166.0	452.0	7.5

≠ Total water added = 14 ml.

* At this point the cultures were removed from the refrigerator and incubated for the remainder of the time at room temperature.

TABLE VII. Plate counts of Rh. meliloti (W_{100}) in an irrigated brown soil from near Lethbridge, treated with dipotassium phosphate, sterilized, inoculated and stored in erlenmeyer flasks stoppered with cotton, half at room temperature and half at 3°C. (millions per gram).

	3° C.			Room Temperature		
Incubation time in days	No nutrients added	0.2% K_2HPO_4	Water loss in gm.	No nutrients added	0.2% K_2HPO_4	Average water loss in gm. *
0	14.9	14.9	0.0	14.9	14.9	0.0
9	6.8	16.0	0.2	958.0	754.0	1.0
51	5.5	16.3	0.9	46.1	60.5	4.4
86	4.3	3.6	1.1	55.1	40.7	6.7
121	2.12	15.9	2.8	1.83	2.40	13.1
162	0.55	2.31	2.8	1.64	7.46	13.8
213	5.00	5.42	3.9	0.38	1.49	16.1
331	0.13	14.0	11.7	0.27	0.84	16.7
550	1.92	3.05	7.6	1.26	0.07	16.6

* Total water added = 18 ml.

legume inoculation cultures. The counts were as high as those obtained in the experiment described in Table II, at least in those cultures held at room temperatures, but it was difficult to detect any consistent trend in the plate counts resulting from the different treatments. However, there did appear to be somewhat higher numbers in those cultures held at room temperature to which sucrose had been added. Of special interest were the high counts on the cultures stored for 402 days in the refrigerator.

One experiment was carried out in which all the cultures were stored in the refrigerator for 54 days, and were then removed and stored for another 65 days at room temperature, and the results are given in Table VI. The same nutrient treatments were used as in the previous experiment (Table V) and similar rather variable results were obtained. Of interest was the fact that the addition of calcium carbonate alone or calcium carbonate plus dipotassium phosphate produced almost immediate increases in numbers of rhizobia present. In cultures to which sucrose had been added there was not a noticeable increase in numbers until after 39 days. After that time, the largest counts, in general, were obtained from those cultures to which sucrose had been added.

The average water loss after 54 days was 1.4 gm.; after 119 days it was 7.5 gm.

In only one experiment were the bacteria grown in a mineral soil alone. In Table VII are given the results of plate counts of Rh. meliloti in a brown irrigated soil from near Lethbridge. The soils of that area were known to be probably low in phosphorus, so dipotassium phosphate was the only treatment added. It did not appear to affect the highest count obtained in the cultures held at room temperature, but appeared to maintain slightly higher numbers over a long period of time. In the refrigerated cultures a higher maximum count was obtained from the cultures treated with K_2HPO_4, and after 550 days the numbers were still higher in the cultures to which phosphate was added.

(b) Results of Experiments with Sodium Nitrate

Table VIII gives results of plate counts of cultures treated with two levels of sodium nitrate and one of sucrose. The nitrate alone appeared to have a stimulating effect on the growth of the bacteria, but the most interesting result obtained was the marked decrease in numbers when sucrose and sodium nitrate were both present. The numbers were always very much lower than when sodium nitrate alone was present, and in three cases no colonies at all of rhizobia were found on the plates. These results were rather a surprise, so two other experiments were set up to obtain further information, and the results are given in Tables IX

TABLE VIII. Plate counts of Rh. leguminosarum (B_3) in peat-black soil mixture, given treatments of sodium nitrate and sucrose, sterilized, inoculated and incubated in 8-ounce brown screw-top glass jars plugged with cotton below the top, at room temperature (millions per gram).

Incubation time in days	No treatments added	250 ppm NO_3-N added	500 ppm NO_3-N added	500 ppm + 2% sucrose added	* Average water loss in gm.
0	7.9	7.9	7.9	7.9	0.0
13	1482.0	577.0	335.0	8.5	0.5
46	187.0	252.5	515.0	214.0	2.1
83	223.4	237.5	702.0	179.0	3.3
112	324.0	207.0	466.0	0.0	4.8
168	109.0	220.0	144.0	134.0	6.9
252	36.4	123.0	171.0	0.0	10.0
336	78.9	21.1	94.1	0.0	12.3
364	32.8	71.1	46.2	8.9	12.0

* Total water added = 35 ml.

and X. The results in Table IX showed that whenever sodium nitrate and either 1.0 or 2.0 per cent sucrose were both added to the medium the growth of the root nodule bacteria was greatly reduced. The numbers were lower when potassium phosphate was present, also. In general, where sodium nitrate alone was added the numbers were higher than in the untreated soil. The effect of adding sucrose alone was unexpected. It may have been because of a relatively high concentration of nitrate nitrogen in the peat used.

Table X represents results of an experiment set up in an attempt to determine the concentration of sucrose at which the growth of rhizobia was seriously affected. The results in Table IX seemed to indicate that concentrations of 1.0% sucrose and 500 ppm nitrogen as sodium nitrate severely limited the growth of the bacteria, so four concentrations of sucrose below that level were tried. In addition, two concentrations of dipotassium phosphate were used with the sodium nitrate.

The results were similar to those previously obtained except that after 119 days there were lower numbers where sodium nitrate alone was added than in the untreated base. Where 0.1% and 0.25% sucrose plus 500 ppm nitrate nitrogen were present, growth of the rhizobia was possible, though the numbers were generally lower than in the untreated soil after eighteen days. Where more than 0.25% sucrose

TABLE IX. Plate counts of *Rh. leguminosarum* (K_1) in peat-black soil mixture, given treatments of sodium nitrate, dipotassium phosphate and sucrose, sterilized, inoculated and incubated in erlenmeyer flasks stoppered with cotton, at room temperature (millions per gram).

Incubation time in days	No treatments added	500 ppm NO_3-N added	500 ppm NO_3-N + 0.2% K_2HPO_4 added	500 ppm NO_3-N + 0.2% K_2HPO_4 + 2.0% sucrose added	500 ppm NO_3-N + 2.0% sucrose added	2% sucrose added	500 ppm NO_3-N + 1% sucrose added	500 ppm NO_3-N + 0.2% K_2HPO_4 + 1% sucrose added	Average water loss in gm. *
0	2.3	2.3	2.3	2.3	2.3	2.3	2.3	2.3	0.0
12	74.0	35.0	34.0	0.0	0.0	0.0	0.0	0.0	0.7
37	13.2	191.0	1.0	65.0	0.0	0.0	4.0	0.0	3.3
91	5.9	0.8	0.0	0.0	0.0	0.0	5.7	0.0	11.9
146	45.4	76.1	14.7	0.0	0.0	0.0	0.0	0.0	16.0
204	3.5	4.6	2.1	0.0	0.0	0.0	0.0	0.0	19.8
235	1.5	2.1	1.9	0.0	0.0	0.0	0.0	0.0	28.2

* Total water added = 31.5 ml.

TABLE X. Plate counts of Rh. leguminosarum (K_1) in peat-black soil mixture, given treatments of sodium nitrate, dipotassium phosphate and sucrose, sterilized, inoculated and incubated in erlenmeyer flasks stoppered with cotton (millions per gram).

Incubation time in days	No treatments added	500 ppm NO_3-N added	500 ppm NO_3-N + 0.1% sucrose added	500 ppm NO_3-N + 0.25% sucrose added	500 ppm NO_3-N + 0.5% sucrose added	500 ppm NO_3-N + 0.75% sucrose added	500 ppm NO_3-N + 0.05% K_2HPO_4 added	500 ppm NO_3-N + 0.1% K_2HPO_4 added	Average water loss in gm. *
0	1.6	1.6	1.6	1.6	1.6	1.6	1.6	1.6	0.0
18	4.0	247.0	280.0	26.8	0.0	0.0	53.0	154.0	1.2
74	31.5	143.0	33.2	2.3	32.6	0.0	13.5	0.1	8.0
119	21.9	0.9	20.9	10.9	0.0	35.0	10.9	171.0	13.8
150	47.0	19.7	3.17	31.5	1.8	11.6	0.8	73.4	14.4
171	71.2	20.2	14.2	34.2	0.0	0.0	73.4	42.2	19.8

* Total water added = 30 ml.

was used, growth was uncertain, and frequently no colonies appeared on the plates, even at the lowest dilution used. Where phosphate but no sucrose was used, growth took place, though it was erratic, varying considerably between cultures having the same treatment.

(c) Results of Experiments with Mineral Oil and Sodium Alginate

These experiments were begun to determine the effect of materials of potential value as "stickers" and preservatives on the growth of rhizobia in moist humus cultures. In Table XI are the results of an experiment in which sodium alginate and mineral oil were added to cultures of Rh. leguminosarum in peat-black soil mixture held at 3°C. In general, the addition of sodium alginate to the culture reduced the numbers considerably. Only one count, of 7.5% sodium alginate at 337 days, was higher than the corresponding count from the untreated soil. However, after 460 days, the numbers in most of the cultures to which sodium alginate had been added had risen to a few hundred millions per gram.

In the cultures to which the mineral oil had been added much greater increases took place. Mostly the numbers were below those present in the untreated soil, but most of the counts obtained after 92 days were above 300 millions per gram. The highest count obtained where mineral oil was present was 1057 millions per gram, with 12% mineral

TABLE XI. Plate counts of Rh. leguminosarum (K_1) in peat-black soil mixture, given treatments of mineral oil and sodium alginate, sterilized, inoculated and stored in erlenmeyer flasks stoppered with cotton at 0 - 3°C. (millions per gram).

Incubation time in days	No treatment added	7.5% Sodium Alginate	15% Sodium Alginate	30% Sodium Alginate	6% Mineral Oil	12% Mineral Oil	24% Mineral Oil	Average water loss in gm. *
0	11.4	11.4	11.4	11.4	11.4	11.4	11.4	0.0
5	5.4	5.2	6.5	4.8	5.7	6.4	6.1	0.1
27	5.2	1.2	2.8	0.4	6.4	33.3	1.4	0.3
50	36.8	1.7	0.0	0.0	5.2	31.9	0.7	0.8
92	368.0	1.6	0.006	0.0	483.8	320.0	73.7	1.6
144	540.0	0.15	50.0	0.27	707.0	840.0	33.8	2.4
224	414.0	0.006	0.0	65.2	826.0	1057.0	405.0	3.6
337	1212.0	3000.0	0.37	6.8	801.0	850.0	1022.0	7.0
460	800.0	250.8	100.0	6.4	765.0	632.0	917.0	9.1
577	975.0	2175.0	365.0	173.0	387.0	404.0	233.0	11.3

* Total water added = 24 ml.

oil present, after 224 days. The highest count in the untreated base was 1212 millions per gram, after 337 days. Where 24% mineral oil was present, there were not as many high counts obtained, the numbers not rising above 100 millions per gram until after 337 days, and beginning to drop again after 460 days. The other experiments in which mineral oil was added are summarized in tables XII, XIII, and XIV. Table XII presents results from an experiment in which rhizobia were grown in peat-black soil mixture in brown glass screw-top bottles. After 179 days the caps were removed from the bottles, which were plugged with cotton, to allow them to dry out. Only on the first count was there any apparent stimulatory effect from the presence of mineral oil. After that, there was no consistent trend towards higher numbers where the mineral oil was present. After the caps were removed, numbers in all cultures dropped sharply, there being very little difference between any of them.

Table XIII gives counts of rhizobia in peat-black soil mixture in clear glass screw-top jars, in which the bacteria were allowed to grow for ten days, at the end of which time half of the cultures were placed in the refrigerator at 3°C. The remainder were held at room temperature. In this experiment there was an apparent stimulatory effect in the cultures where mineral oil was present, especially where the cultures were refrigerated. The reason for this

TABLE XII. Plate counts of Rh. leguminosarum (K_1) in peat-black soil mixture given treatments of mineral oil, sterilized, inoculated and stored in 8-ounce brown screw-top glass jars at room temperature (millions per gram).

Incubation time in days	No treatments added	6% Mineral Oil	12% Mineral Oil	24% Mineral Oil	Average water loss in gm. *
0	10.6	10.6	10.6	10.6	0.0
9	387.0	555.0	235.0	446.0	0.6
53	-	138.0	86.0	92.0	2.6
128	66.0	21.0	19.0	255.0	8.1
179 ≠	34.3	17.2	13.3	7.0	9.2
263	3.5	0.03	1.8	0.1	30.5
291	0.19	1.5	0.01	0.18	32.8

* Total water added = 35.5 ml.

≠ Tops were removed after 179 days to allow the cultures to dry out more rapidly.

TABLE XIII. Plate counts of Rh. leguminosarum (millions per gram) in peat-black soil mixture, given treatments of mineral oil, sterilized, inoculated and incubated in 8-ounce clear glass screw-top jars. After 10 days, half of the cultures were placed in the refrigerator at about 3°C. and the remainder left at room temperature.

	Room temperature					Refrigerated (3°C.)				
Incubation time in days	No treatments added	6% mineral oil	12% mineral oil	24% mineral oil	Average water loss in gm. *	No treatments added	6% mineral oil	12% mineral oil	24% mineral oil	Average water loss in gm. *
0	2.1	2.1	2.1	2.1	0.0	2.1	2.1	2.1	2.1	0.0
10	75.0	518.0	605.0	566.0	1.2	41.0	650.0	636.0	549.0	1.5
54	465.0	255.0	606.0	597.0	6.8	314.0	346.0	218.0	647.0	2.1
87	295.0	386.0	90.0	76.4	9.0	2.0	634.0	301.0	812.0	2.3
118	180.0	249.0	1.5	49.5	14.5	304.0	398.0	177.0	1059.0	3.6

* Total water added = 50.5 ml.

TABLE XIV. Plate counts of Rh. meliloti (W_{100}) and Rh. trifolii (W_{239}) in peat-black soil mixture, given treatments of mineral oil and sucrose, sterilized, inoculated and stored in 8-ounce clear glass screw-top jars at room temperature (millions per gram).

Incubation time in days	No treatments added	12% mineral oil	24% mineral oil	24% mineral oil + 0.5% sucrose	No treatments added	12% mineral oil	24% mineral oil	24% mineral oil + 0.5% sucrose	Average water loss in gm.*
0	36.0	36.0	36.0	36.0	5.0	5.0	5.0	5.0	0.0
18	229.0	495.0	602.0	104.0	565.0	870.0	438.0	221.0	1.9
54	440.0	27.0	634.0	451.0	212.0	568.0	282.0	1226.0	5.4
82	330.0	265.0	137.0	581.0	400.0	507.0	86.5	495.0	8.2
103	198.0	3.1	128.0	113.0	20.0	65.0	65.5	610.0	12.6

* Total water added = 50.5 ml.

was not clear, especially since where 12% mineral oil was added, the counts were nearly always lower than where either 6% or 24% mineral oil was used.

Since Rh. leguminosarum had been used in all previous experiments but one it was felt that cultures of other species of rhizobia should be tried in jars, in the presence of mineral oil. Table XIV gives the results of this experiment. These were much as expected, though somewhat erratic: at certain times, there was an apparent stimulating effect where the mineral oil was added, and at other times there was an apparent depression. However, the numbers were well maintained throughout the experiment.

Sucrose (0.5%) was added to some of the cultures in this experiment. There appeared to be a certain stimulating effect due to its presence, but it was not possible to carry on the experiment long enough to be sure of this.

(d) Results of Experiments with Refrigerated Cultures

The results of these experiments are given in Tables V, VI, VII, XI and XIII. The results were again much as expected. Larger numbers of bacteria were, as a rule, maintained for a greater length of time. Where the bacteria were inoculated into the medium and immediately placed in the refrigerator, the increase in numbers was much slower than in corresponding cultures held at room temperature. This may be seen in Tables V and VII. It was also observed

that the maximum numbers found were not as great when the cultures were refrigerated in this manner.

One of the more outstanding effects of refrigeration was on the water loss, as seen in Tables V, VII and XIII. In comparison with similar cultures held at room temperature, the refrigerated cultures lost water much less rapidly, even after several months. The small amount of the added water lost may also be seen in Tables VI and XI.

In Table XIII are given results of plate counts of cultures placed in the refrigerator after the numbers of bacteria had become relatively high. After 87 days, the drop in numbers in the unrefrigerated cultures as compared to the refrigerated cultures was easily noticeable. The water loss was also correspondingly greater. After 118 days the difference was even more marked.

(e) Results of Experiments with Screw-top Glass Jars

Two types of jar were used, one a brown jar with a relatively narrow mouth, and the other a clear glass jar with a wider mouth. The brown jars were plugged with cotton below the cap, with the intention of preventing contamination, but it was found that the plugs absorbed moisture after a time and provided an excellent support for mould growth. The clear jars were not plugged with cotton.

The results of these experiments are given in Tables IV, V, VIII, XII, XIII and XIV. While no direct comparison with the growth in erlenmeyer flasks was possible under the conditions of the experiment, it may easily be seen that the numbers of bacteria in the jars were maintained at a level well above Hedlin's (31) standard for minimum numbers in an effective inoculation culture.

The problem of contamination was more serious when the brown jars were used, because of the moisture absorbed by the cotton plug. However, some trouble was experienced with contamination in the erlenmeyer flasks stoppered with rubber stoppers wrapped with cotton. It seems likely that the jars, if properly stoppered, should not be much more susceptible to contamination than a container or culture vessel plugged in the orthodox manner. Furthermore, it was noted that contamination was very much less frequent when the cultures were stored in the refrigerator.

(2) Oxygen Uptake of Rh. Meliloti in Soils.

The results of these experiments are presented graphically. The number of days that the soil was incubated in the presence of straw or alfalfa before it was sterilized and inoculated with rhizobia varied. It was not possible to make comparisons between soils or between different determinations on the same soil, since it was not possible to use the same numbers of bacteria as inoculum for each determination. An attempt was made to determine the weight of organisms introduced in the inoculum, but this was not found possible. The weight of organisms was so small that the determination was probably considerably in error, and in addition the period of observation was long enough so that growth of the bacteria should have been considerable. Hence it was not considered valid to reduce the data to oxygen uptake per unit weight of bacteria in the inoculum. However, some comparisons are possible.

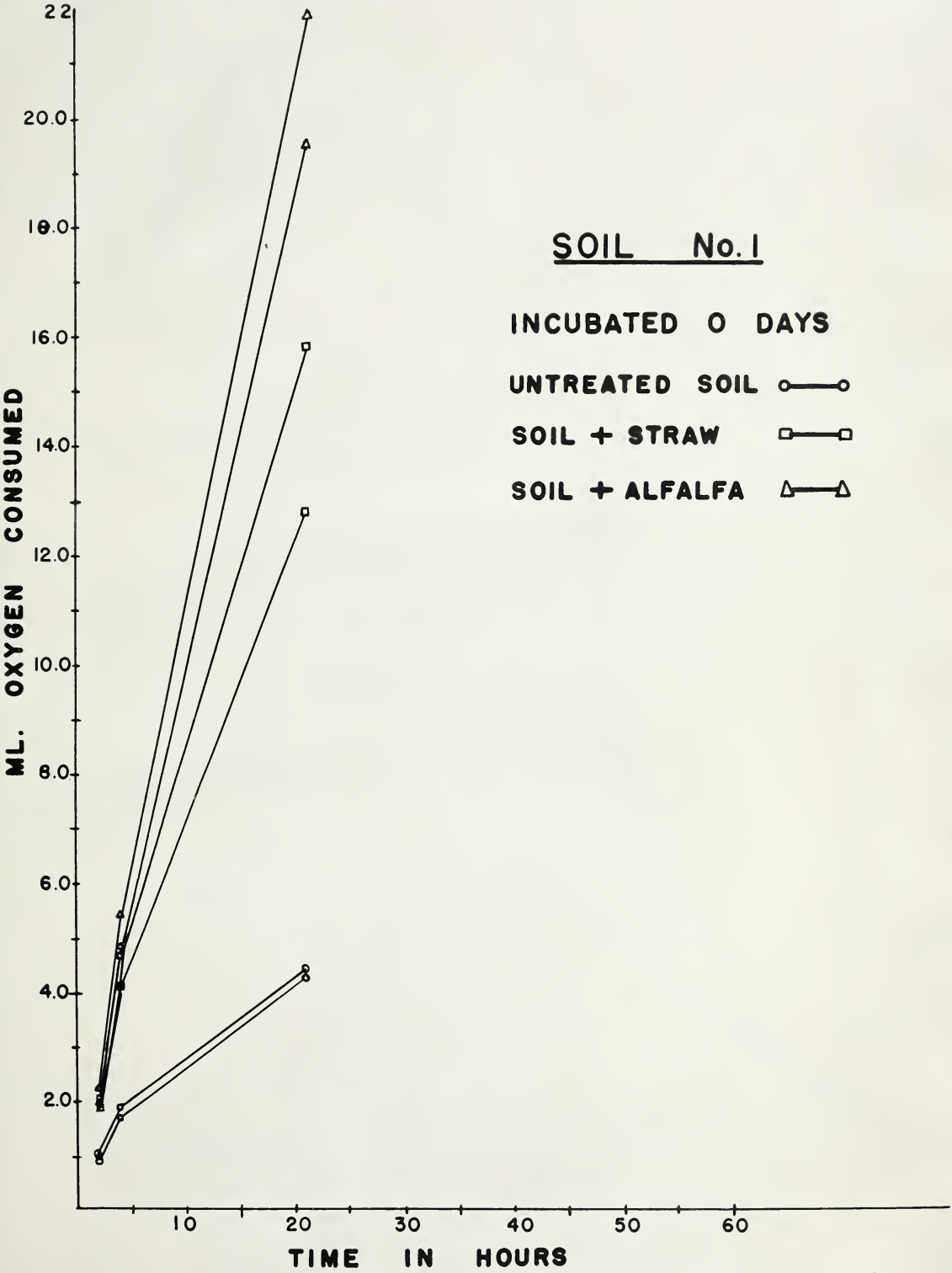
As a general rule, where alfalfa or straw was added to the soil and the mixture moistened, sterilized and inoculated immediately, the oxygen uptake where straw was added was about 12-14 ml. in about 23 hours, and where alfalfa was added, about 20-22 ml. In soil number five, however, the oxygen uptake where straw was added was about 18 ml.

Soil number two did not follow this general pattern. Oxygen uptake where straw was added was below that

of the untreated soil, and where alfalfa was added it was even lower. However, after the soil had been incubated with straw or alfalfa for 10 - 66 days, these observations were reversed.

The general trend was for the increase in oxygen uptake over the untreated soil to decrease as the incubation time before inoculation with rhizobia and measurement of oxygen uptake was increased from 0 to 66 days. When the period of incubation prior to drying, sterilization and inoculation was relatively long, soils number one, number two, number three, and number four showed greater oxygen uptake where straw had been added to the soil than where alfalfa was added. This was not the case with soils numbers five and six. The oxygen uptake for all cultures was generally lower in soil number three, and highest in soil number five. Soil number three was a loam, number two a silt loam, and the others were heavier soils, silty clay loams or clays. Soil number five was a silty clay which had been farmed very well for about fifty years.

The oxygen uptake for the other four soils was about seven to nine ml. after approximately 45 hours in the respirometer. In soil number two the oxygen uptake was about as high at the end of the period of observation as for soils one, four and six, but was lower earlier in the observation period. The difference in oxygen uptake between treatments was greater after ten days incubation before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UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
TIME IN HOURS
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18.0
16.0
14.0
12.0
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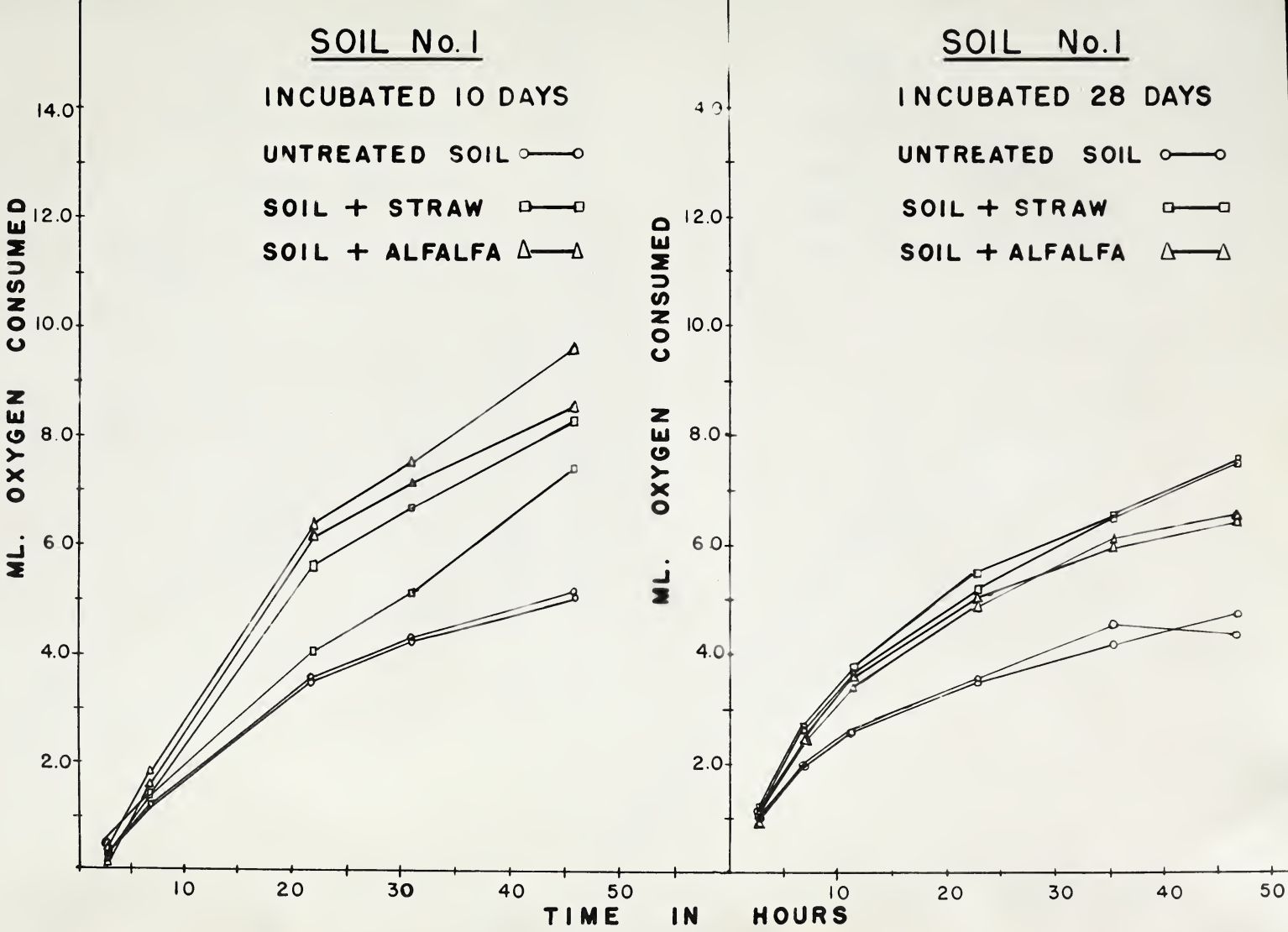
40
50
60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UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
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10.0
8.0
6.0
4.0
2.0
10
20
30
40
50
TIME IN HOURS
SOIL No. 1
INCUBATED 28 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
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4.0
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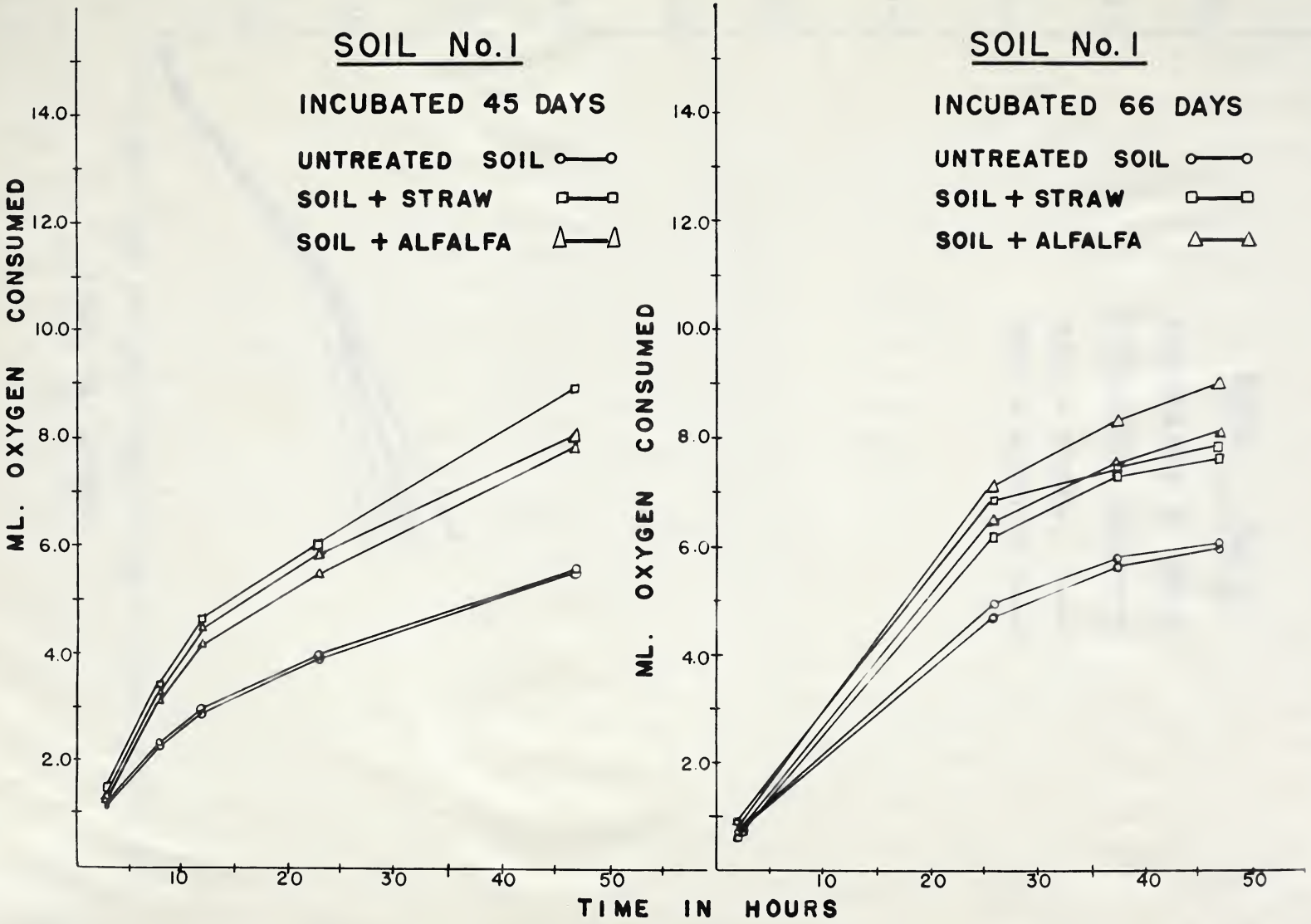
10
20
30
40
50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2.0
10
20
30
40
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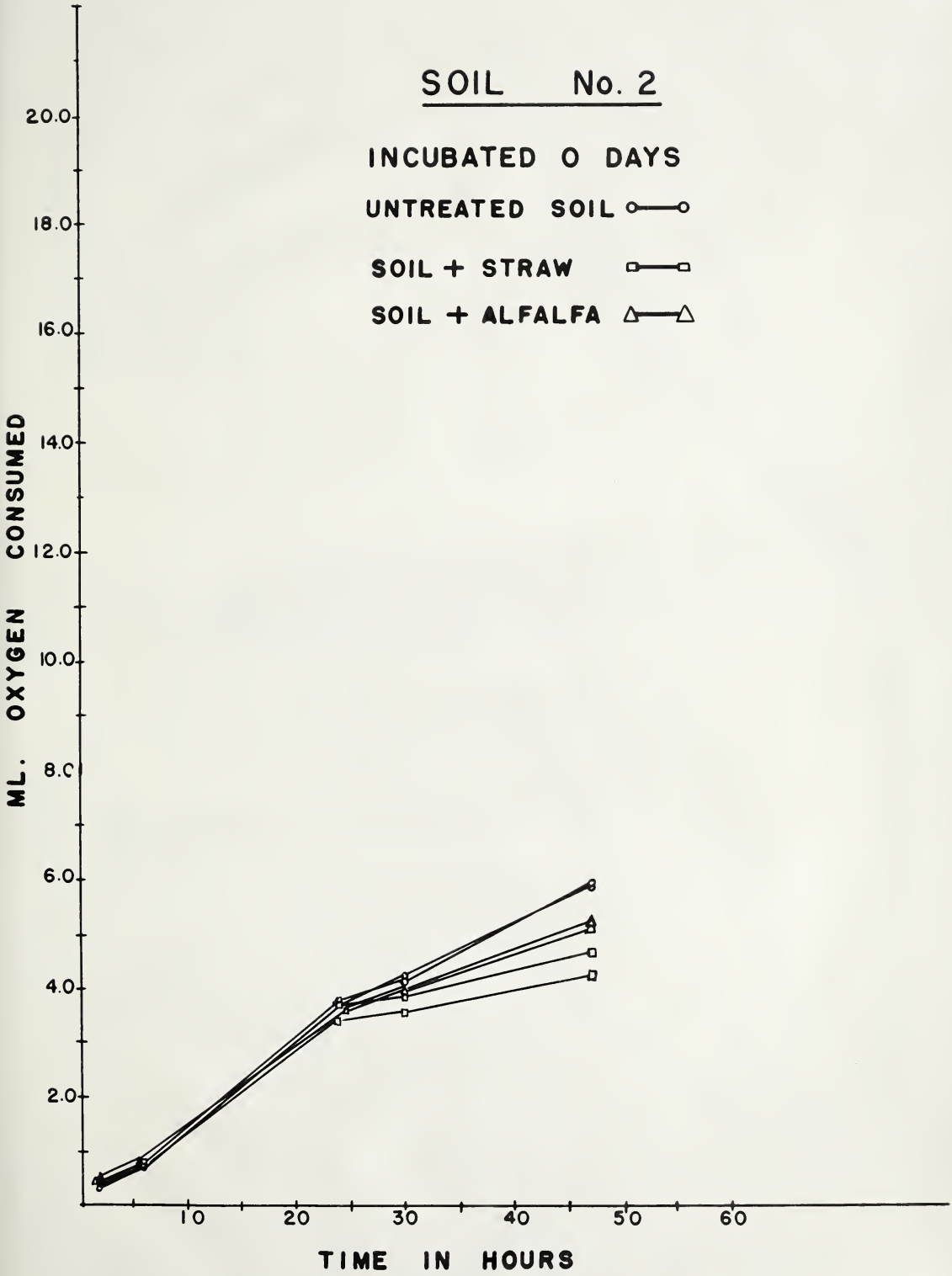
INCUBATED 66 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
TIME IN HOURS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SOIL + ALFALFA
ML. OXYGEN CONSUMED
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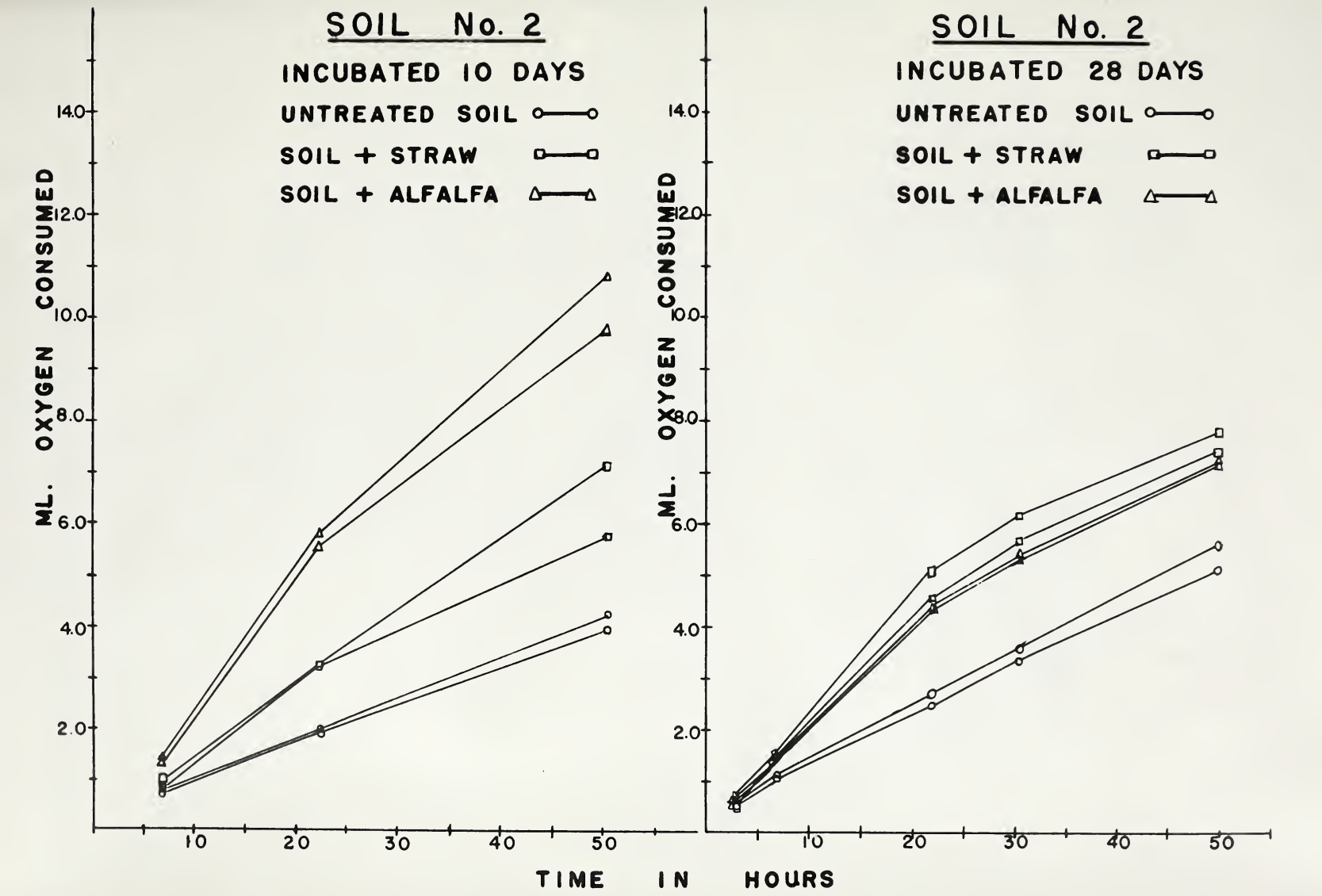
30
40
50
60
TIME IN HOURS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SOIL + ALFALFA
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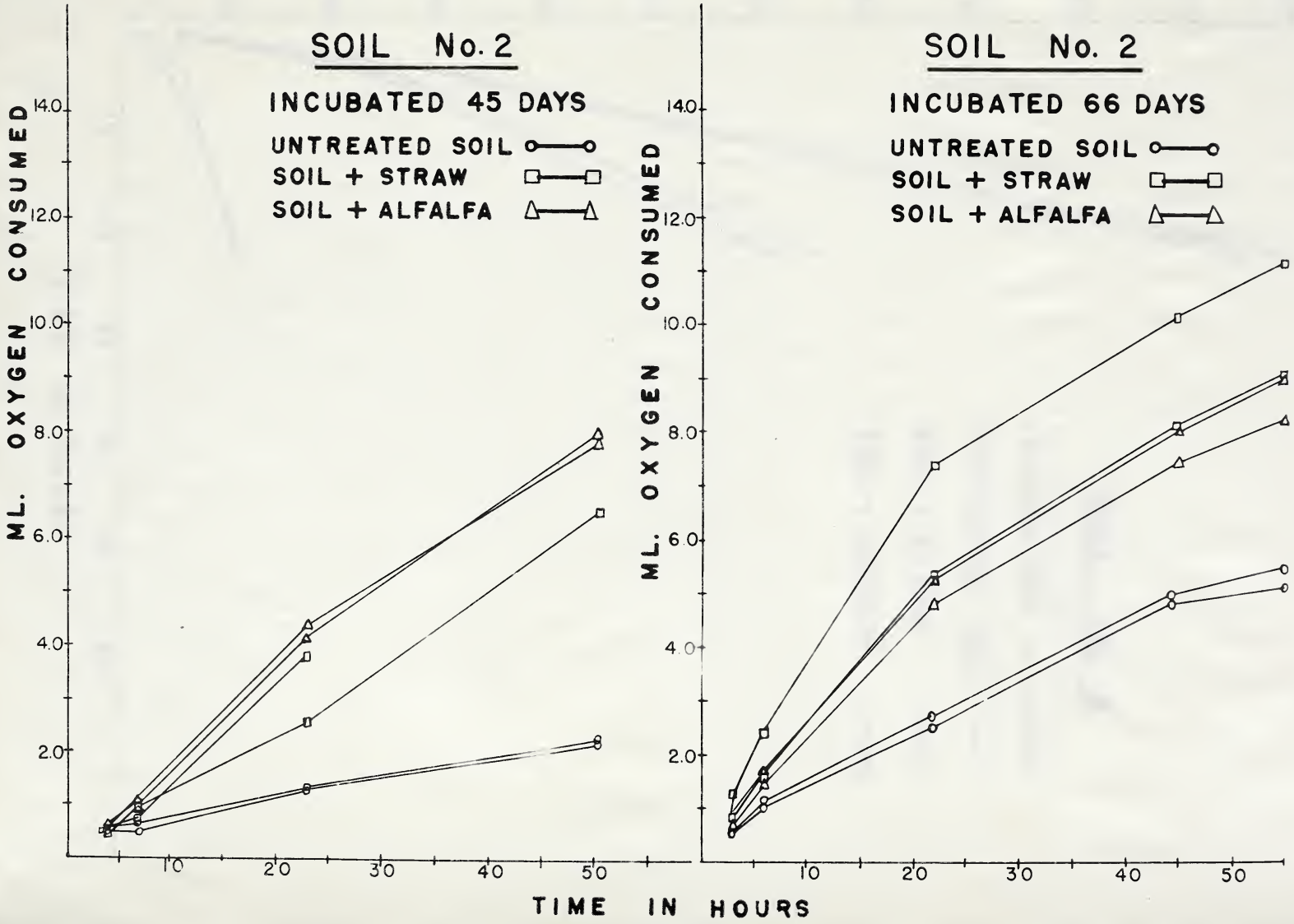
8.0
6.0
4.0
2.0
10
20
30
40
50
SOIL No. 2
INCUBATED 28 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
TIME IN HOURS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6.0
4.0
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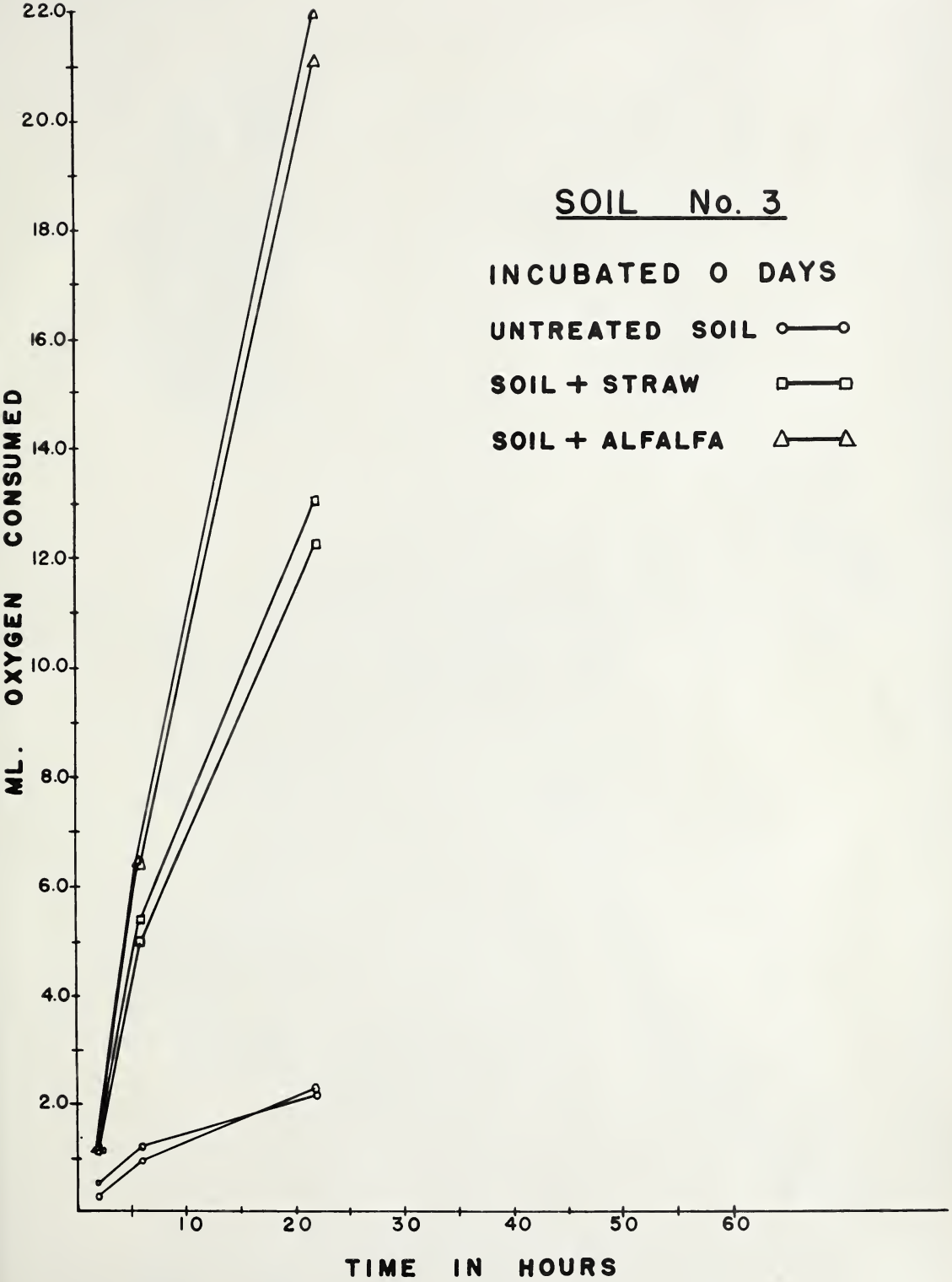
INCUBATED 66 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
TIME IN HOURS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SOIL + ALFALFA
ML. OXYGEN CONSUMED
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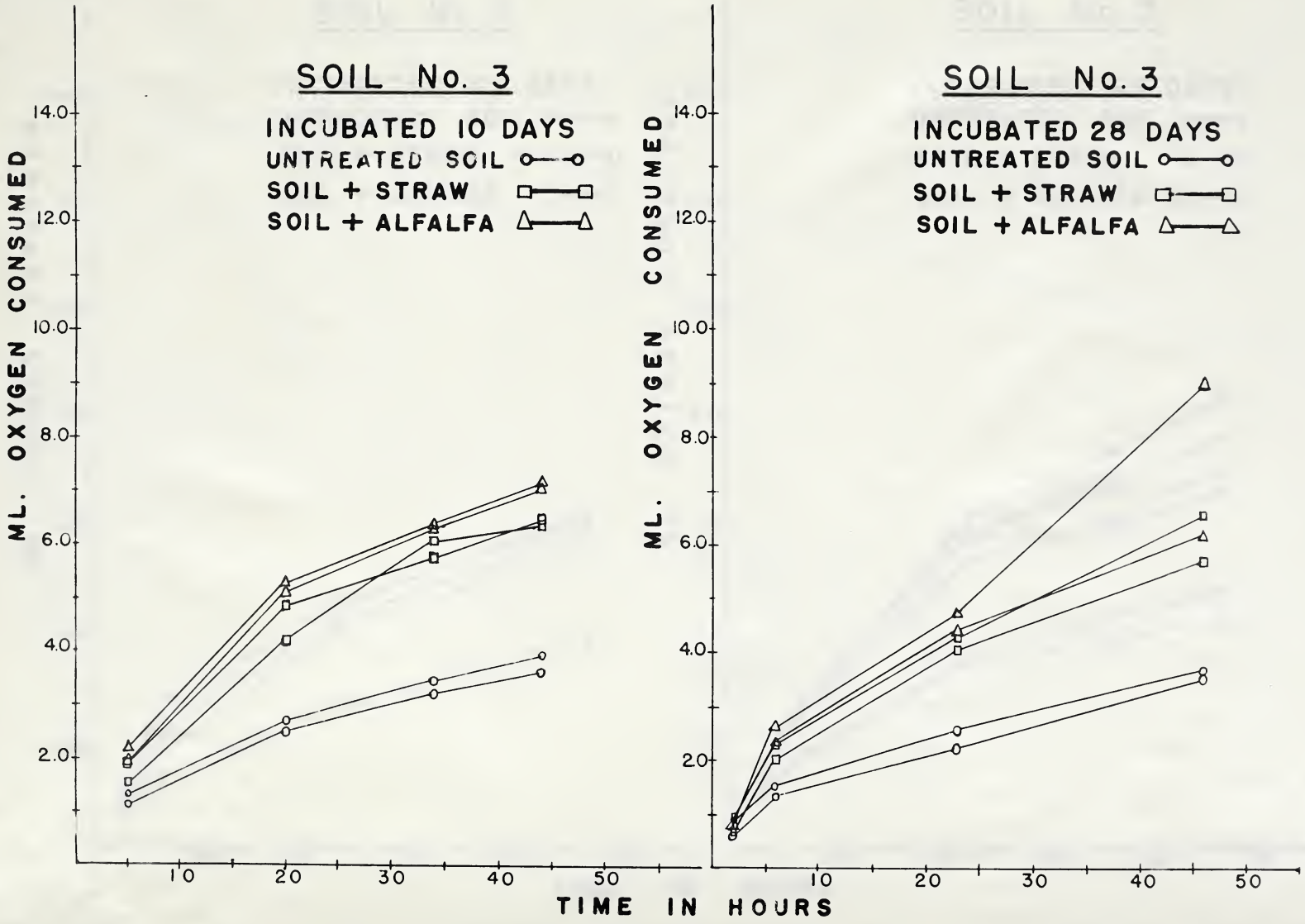
2.0
10
20
30
40
50
60
TIME IN HOURS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SOIL + ALFALFA
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10.0
8.0
6.0
4.0
2.0
10
20
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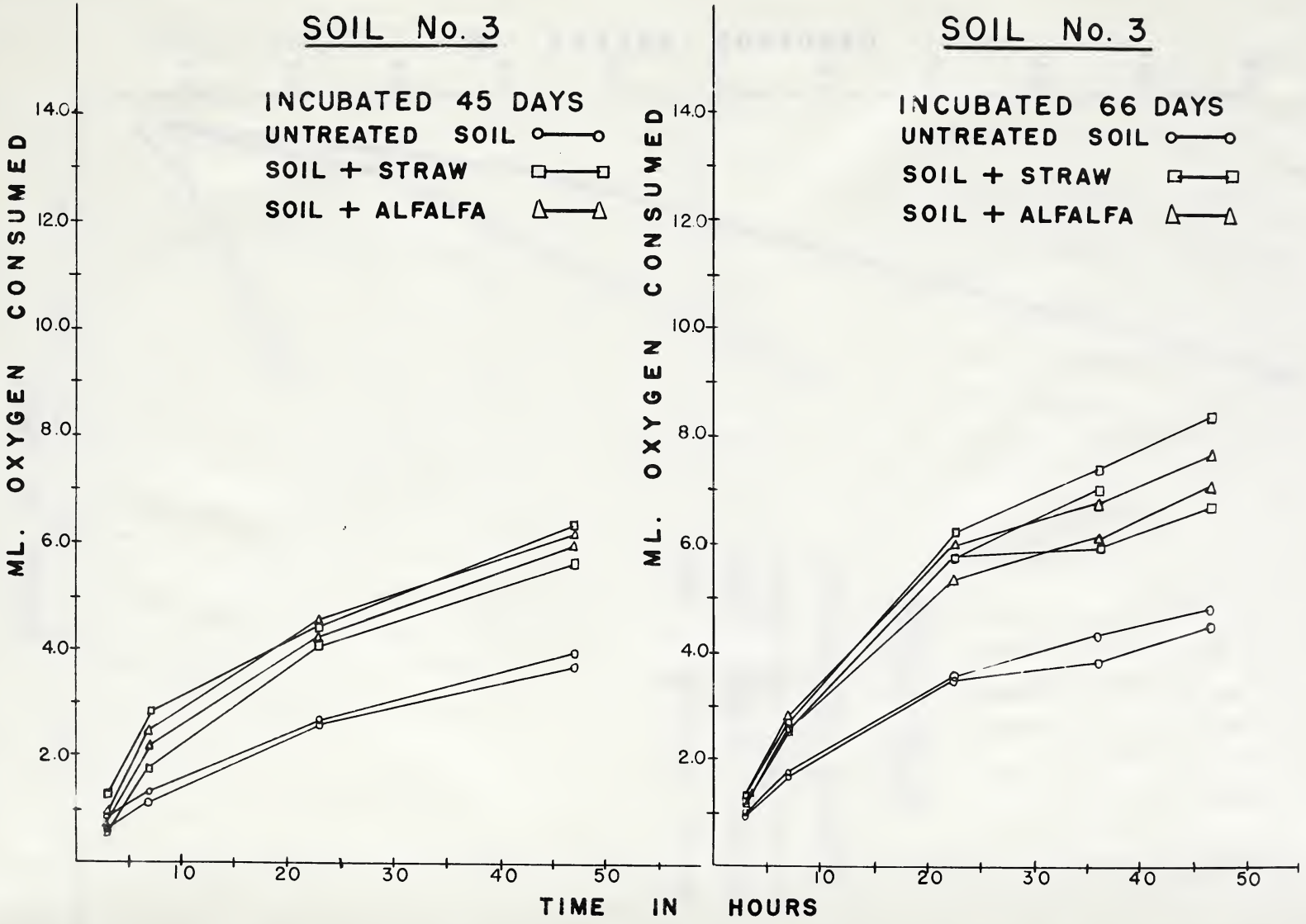
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
TIME IN HOURS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UNTREATED SOIL
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SOIL + ALFALFA
ML. OXYGEN CONSUMED
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8.0
6.0
4.0
2.0
10
20
30
40
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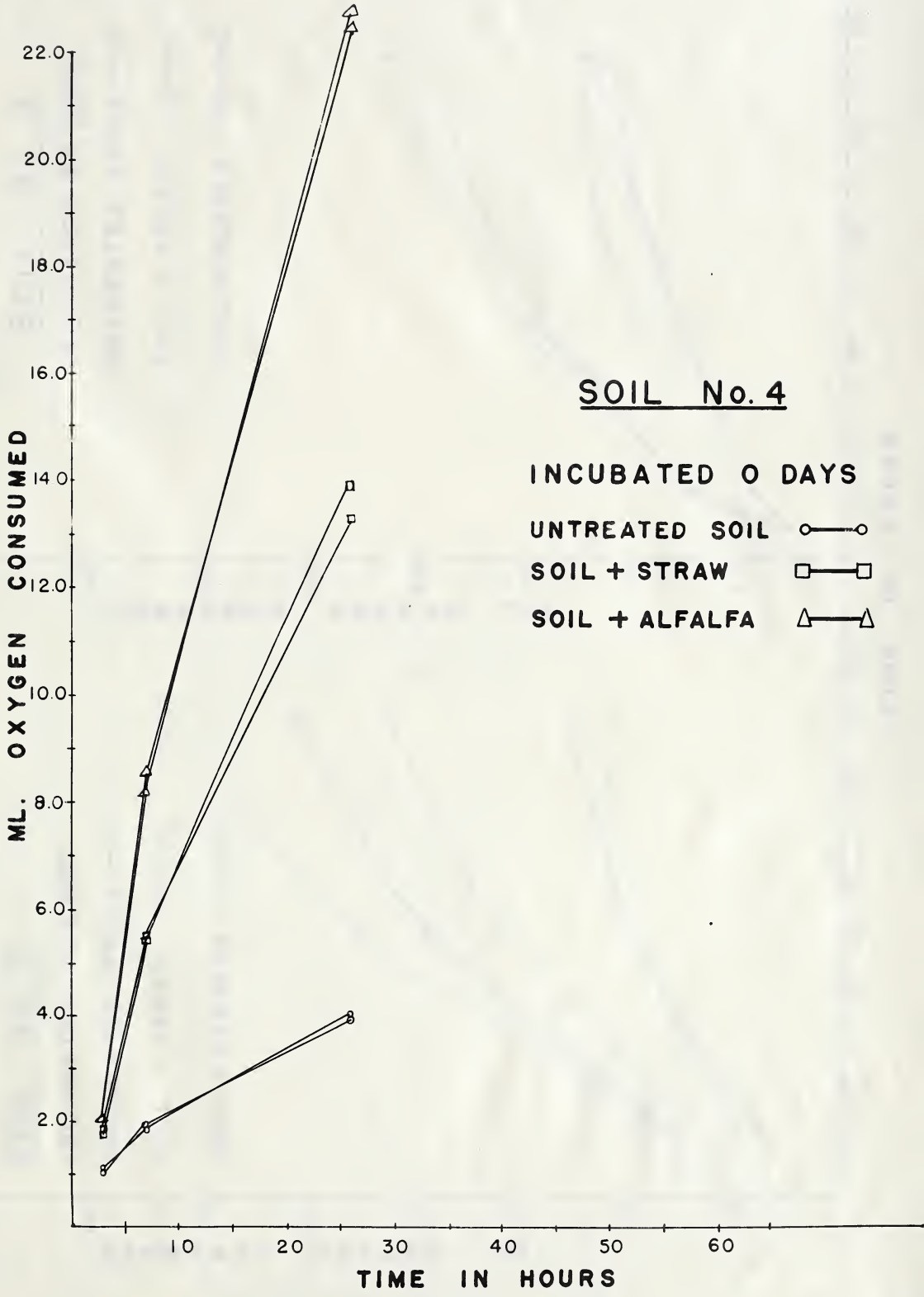
SOIL No. 3
INCUBATED 66 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
TIME IN HOURS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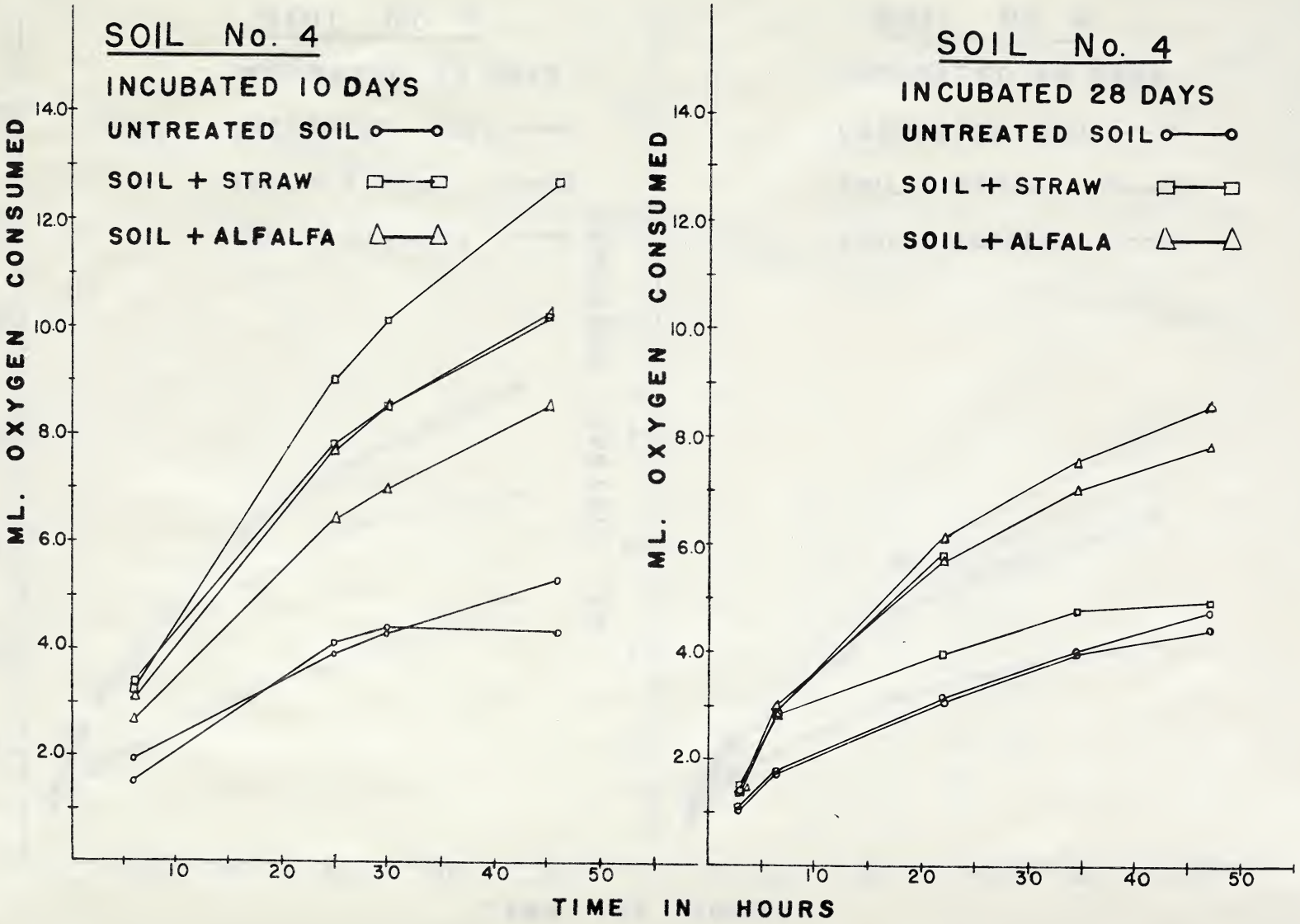
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
22.0
20.0
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
10
20
30
40
50
60
TIME IN HOURS


SOIL No. 4
INCUBATED 10 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
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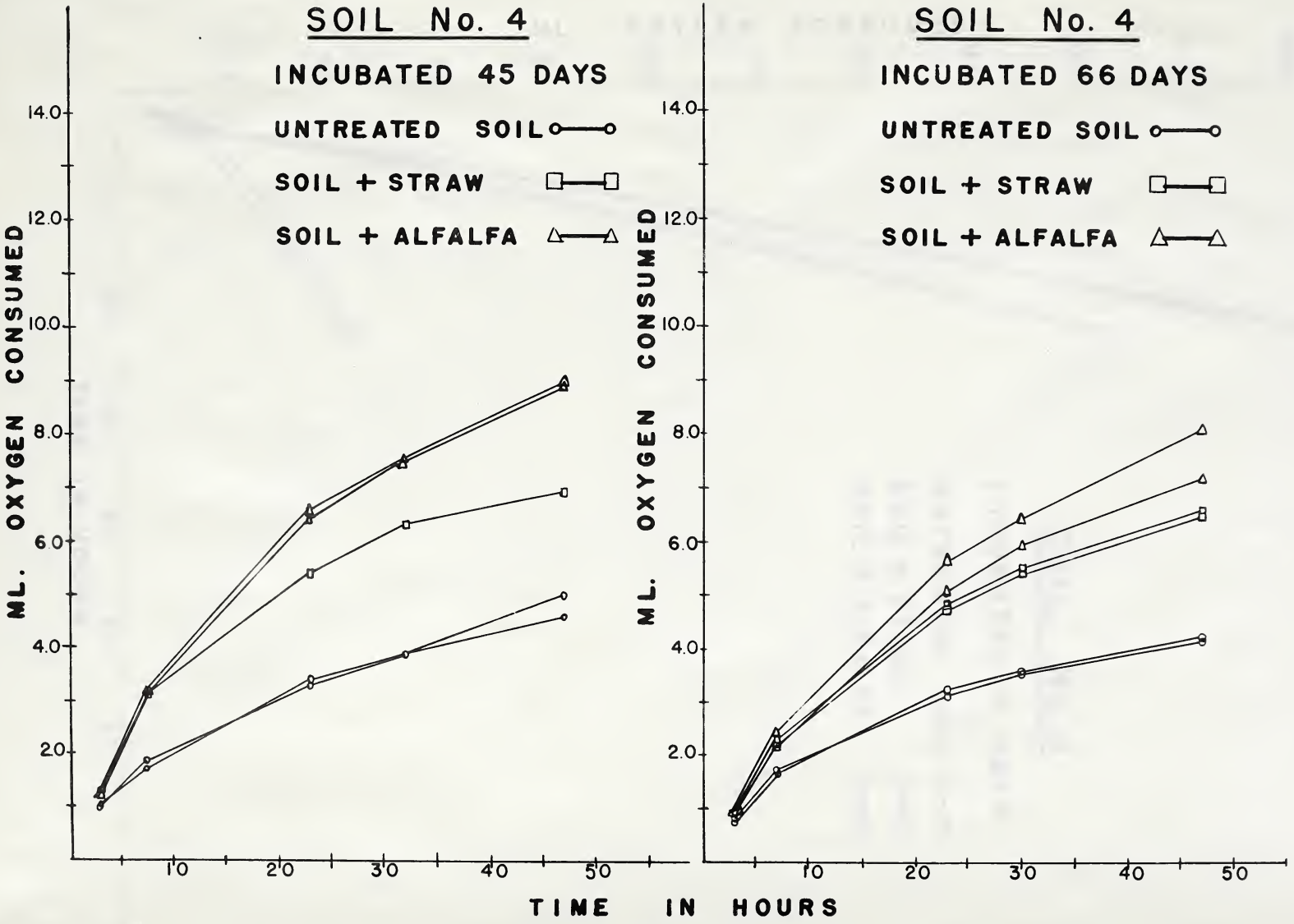
12.0
10.0
8.0
6.0
4.0
2.0
10
20
30
40
50
SOIL No. 4
INCUBATED 28 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALA
ML. OXYGEN CONSUMED
TIME IN HOURS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UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
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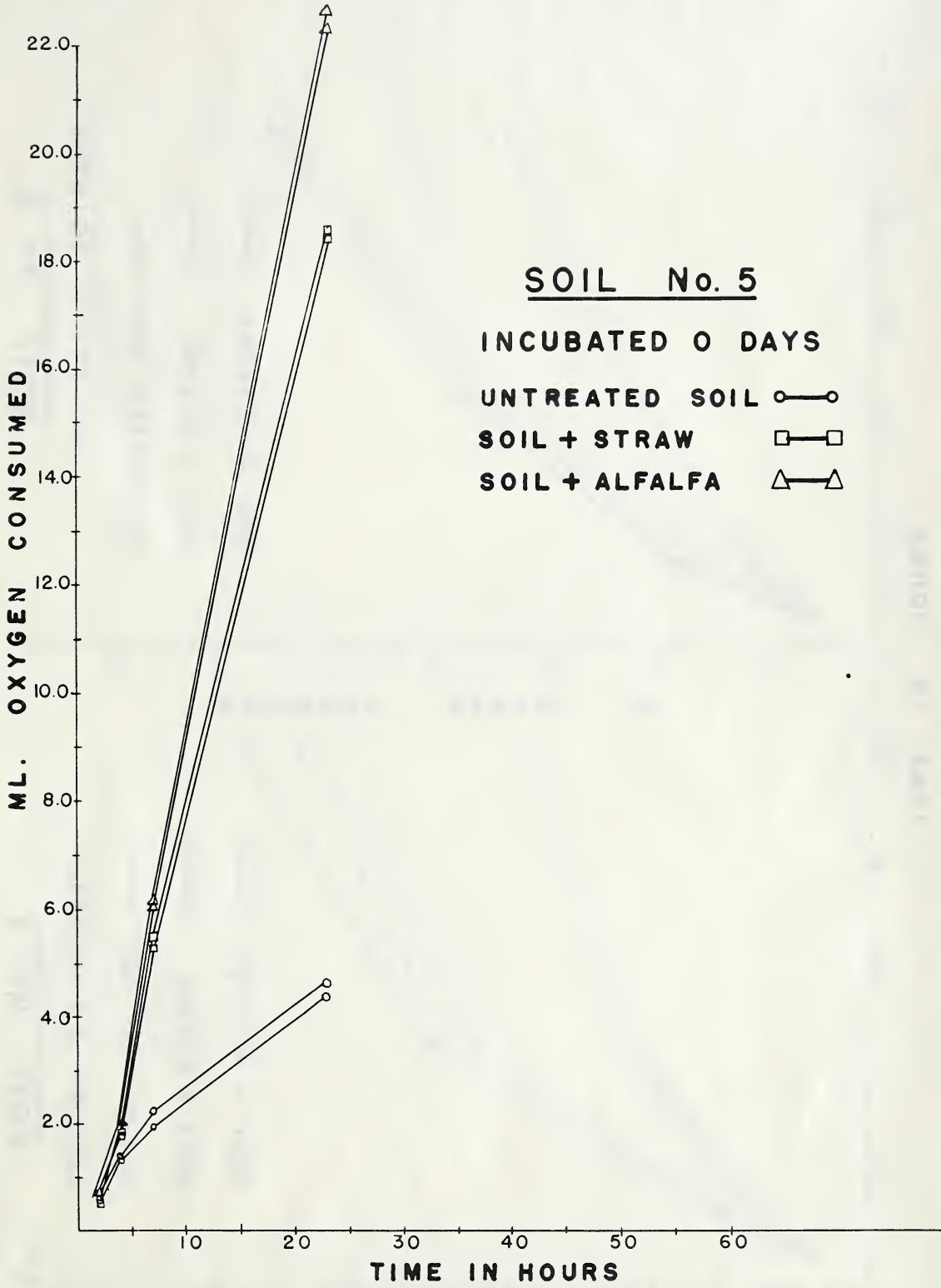
10.0
8.0
6.0
4.0
2.0
1'0
2'0
3'0
4'0
5'0
SOIL No. 4
INCUBATED 66 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
14.0
12.0
10.0
8.0
6.0
4.0
2.0
10
20
30
40
50
TIME IN HOURS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INCUBATED 0 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
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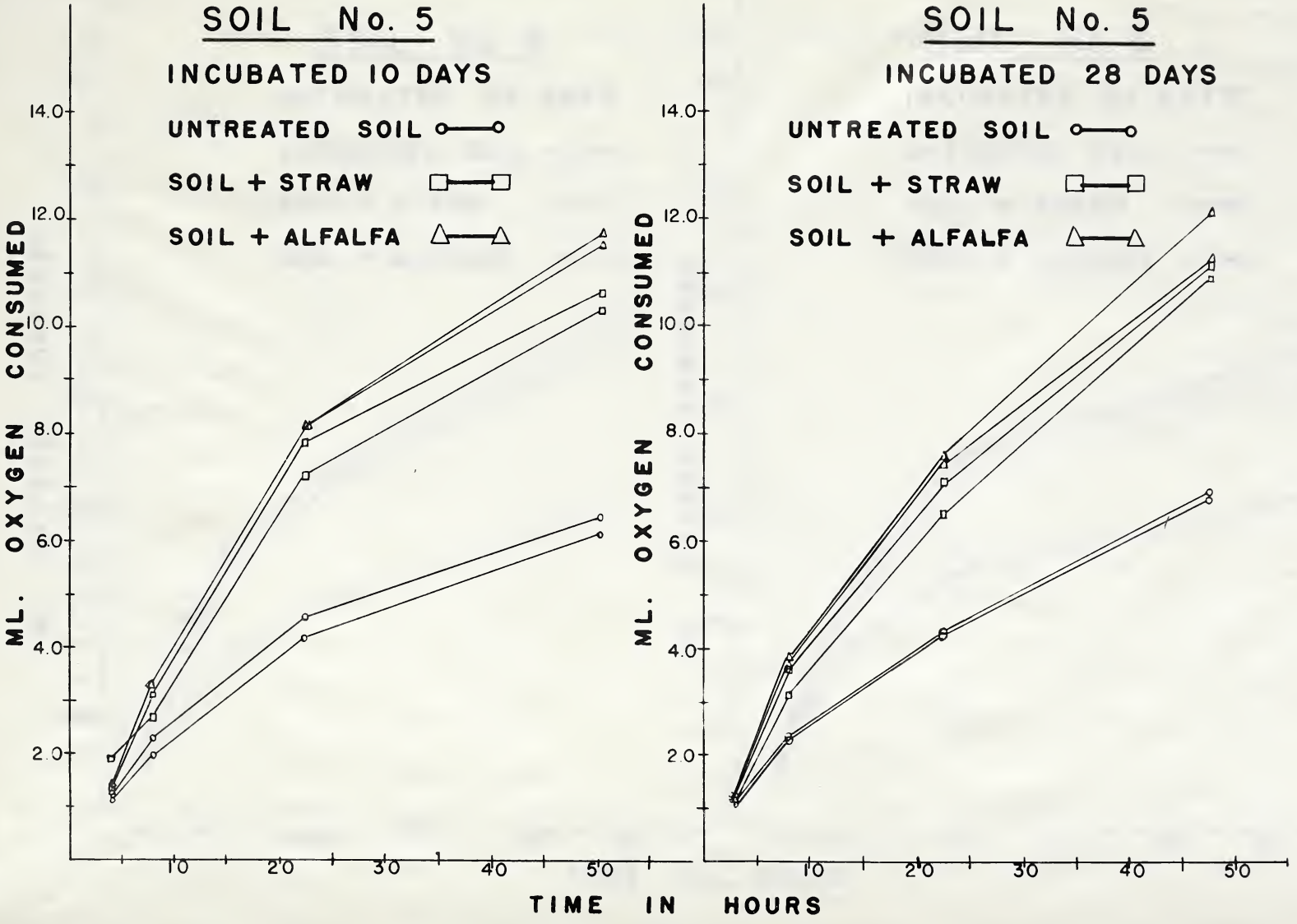
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
10
20
30
40
50
60
TIME IN HOURS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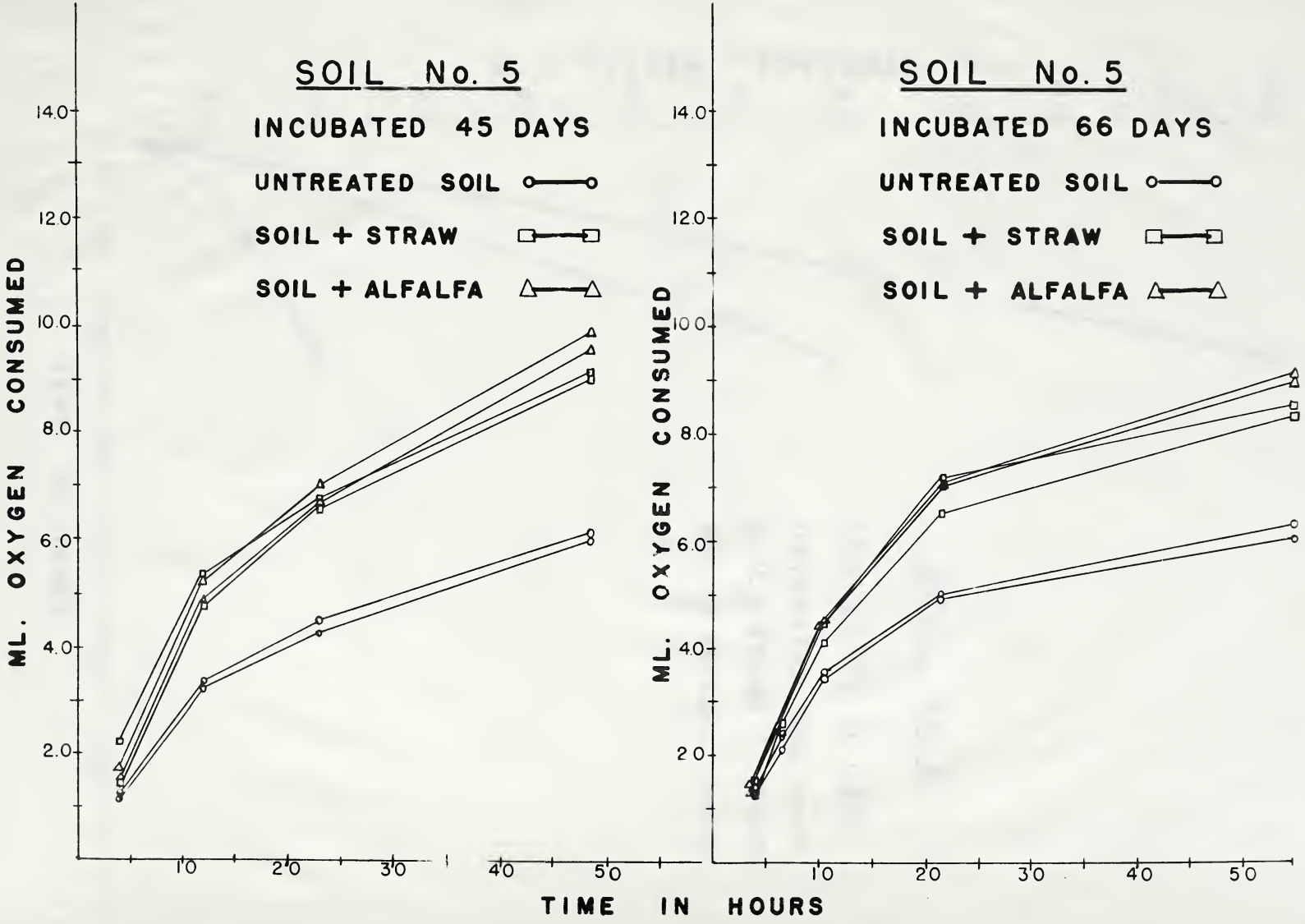
INCUBATED 10 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
14.0
12.0
10.0
8.0
6.0
4.0
2.0
1'0
2'0
3'0
4'0
5'0
SOIL No. 5
INCUBATED 28 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
TIME IN HOURS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UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
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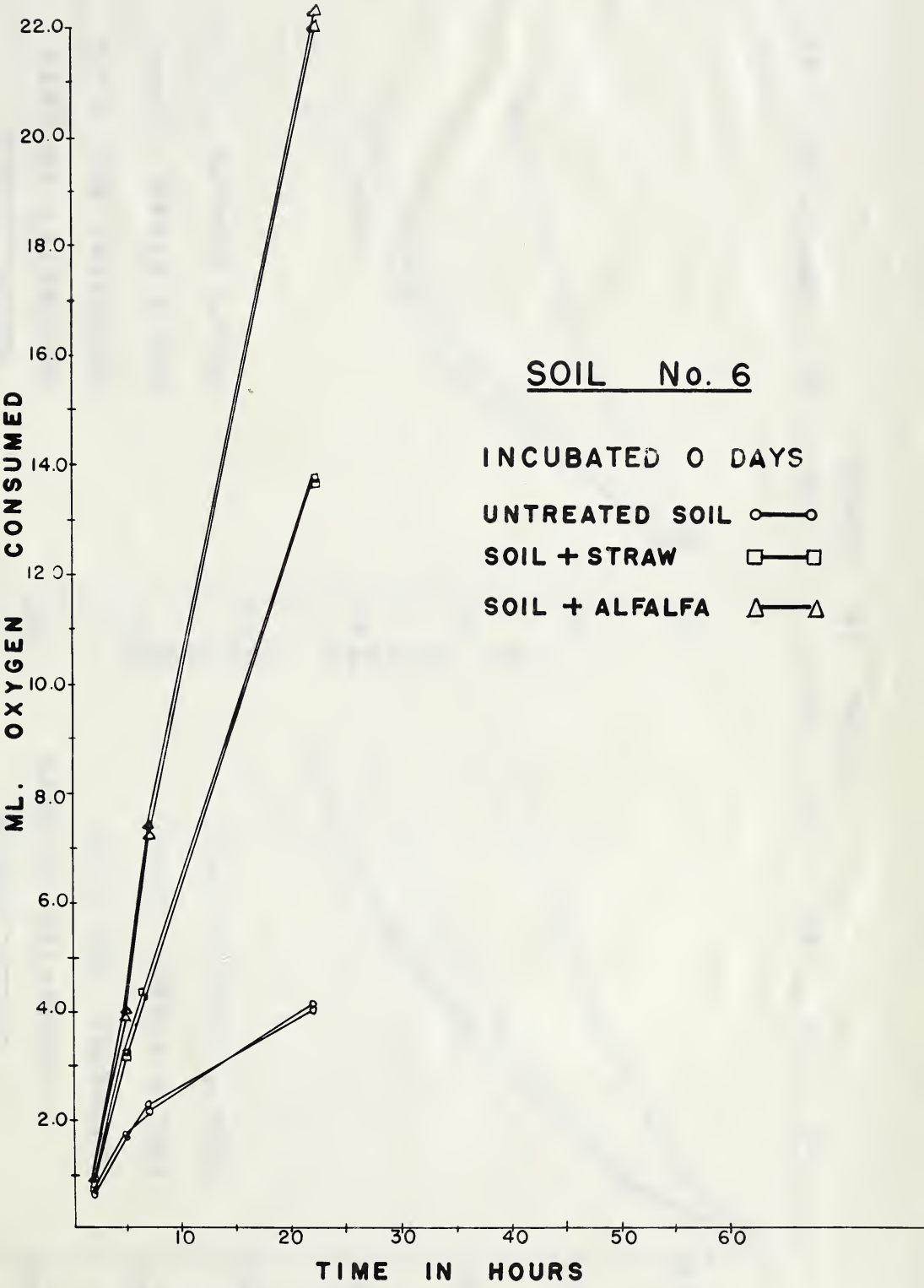
12.0
10.0
8.0
6.0
4.0
2.0
10
20
30
40
50
SOIL No. 5
INCUBATED 66 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
TIME IN HOURS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UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
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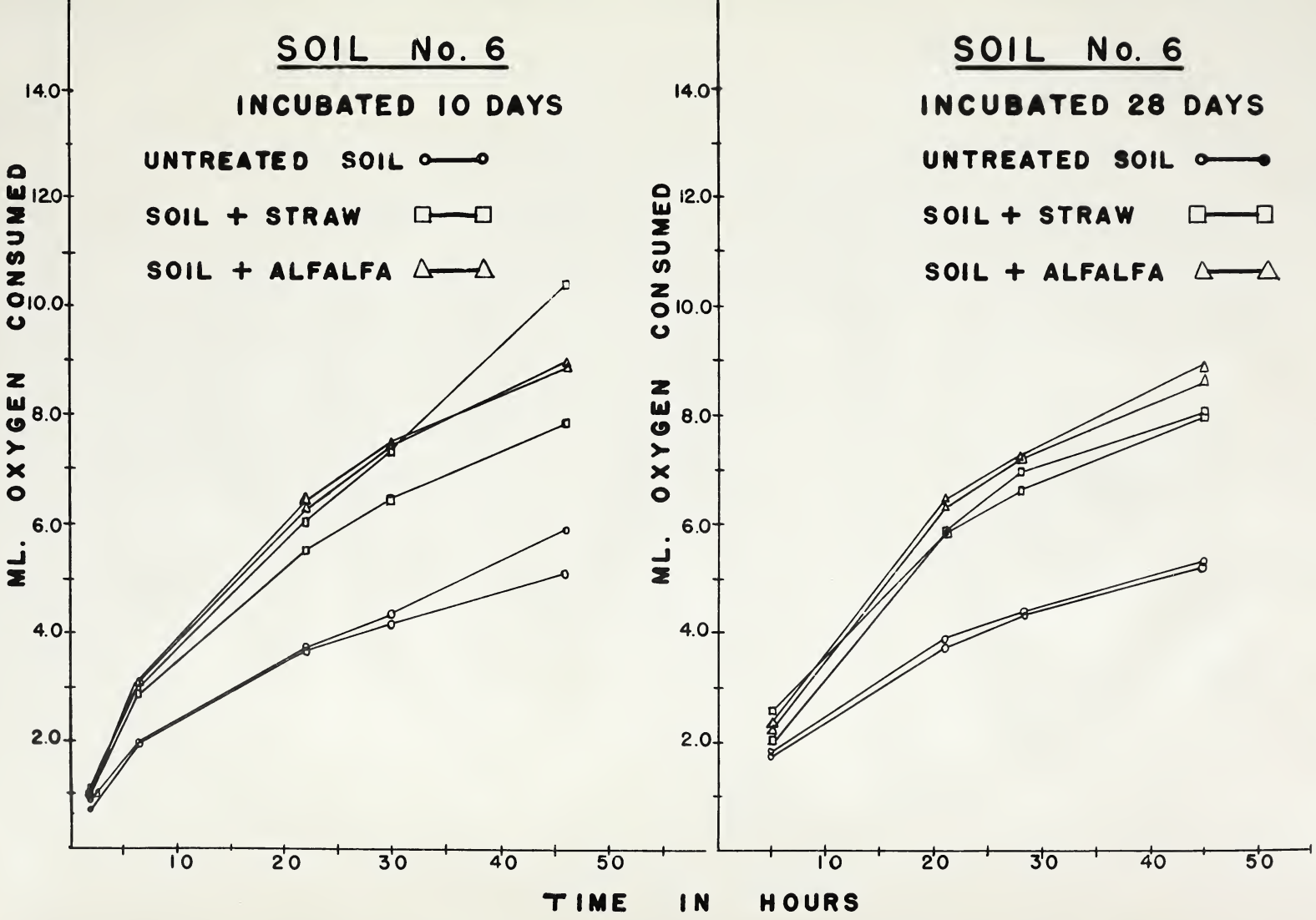
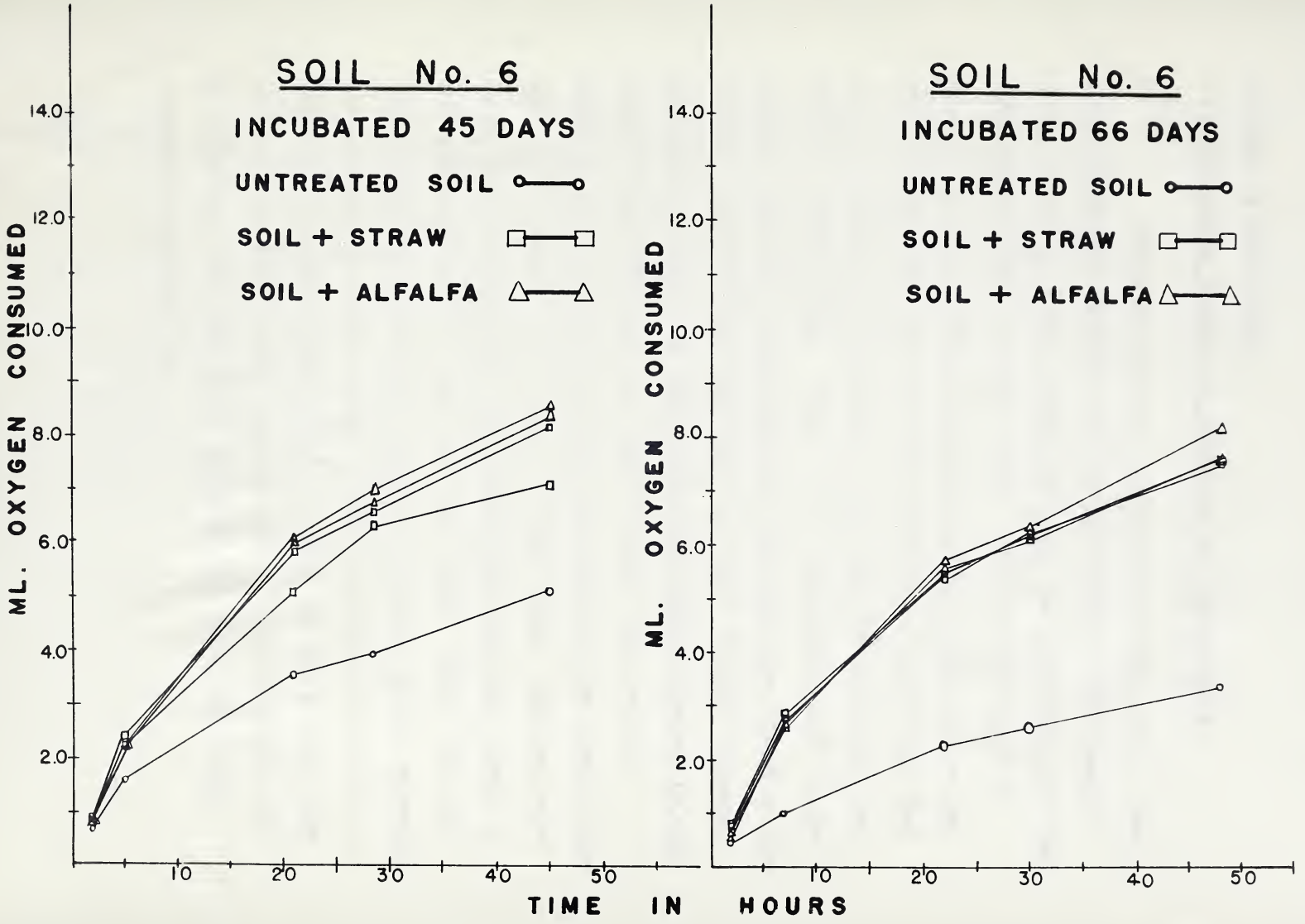
18.0
16.0
14.0
12.0
10.0
8.0
6.0
4.0
2.0
10
20
30
40
50
60
TIME IN HOURS


SOIL No. 6
INCUBATED 10 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
14.0
12.0
10.0
8.0
6.0
4.0
2.0
10
20
30
40
50
SOIL No. 6
INCUBATED 28 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
TIME IN HOURS


SOIL No. 6
INCUBATED 45 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
14.0
12.0
10.0
8.0
6.0
4.0
2.0
10
20
30
40
50
TIME IN HOURS
SOIL No. 6
INCUBATED 66 DAYS
UNTREATED SOIL
SOIL + STRAW
SOIL + ALFALFA
ML. OXYGEN CONSUMED
14.0
12.0
10.0
8.0
6.0
4.0
2.0
10
20
30
40
50

drying in this soil.

(3) Dilution Counts of Rhizobia in Field Soils

These results are presented in Table XV. Good nodulation was obtained in all dilutions of the sample from the H. A. Craig farm, and from the east field of the University Animal Science farm. Nodules were poor or absent in the 1:100,000 dilution of the sample from the University Soils Farm and all dilutions higher than the 1:100 dilution of the sample from the west field of the Animal Science Farm. Hence it was concluded that in the samples from the Craig farm and from the east field of the Animal Science Farm there were at least 100,000 rhizobia per gram of soil, in the Soils farm sample between 10,000 and 100,000 rhizobia per gram of soil, and probably between 100 and 1,000 rhizobia per gram in the sample from the west field of the Animal Science Farm. Nodules were found on one check pot, but conditions were not ideal in the greenhouse where the pots were kept. While the pots were always watered with sterile distilled water, whenever the weather was warm enough outside to allow the snow on the roof to melt there would be water dripping into some of the pots from cracks in the roof. This certainly did not improve the chances of keeping the pots free from outside contamination.

TABLE XV. Dilution counts of Rh. meliloti in four alfalfa field soils in the vicinity of Edmonton. Results are expressed as nodulation (+), slight nodulation (⊕), or no nodulation (-) in each of three pots of each dilution.

Dilution	H.A. Craig Farm	Dept. of Soils Farm	Animal Science Farm, West Field	Animal Science Farm, East Field	Check (Uninoculated)
1:10	+ + +	+ + +	+ ⊕ ⊕	+ + +	
1:100	+ + +	+ + +	⊕ + +	+ + +	
1:1000	+ + +	+ + +	⊕ ⊕ ⊕	+ ⊕ +	
1:10,000	+ + +	+ + +	⊕ ⊕ -	+ + +	
1:100,000	+ + ⊕	- - ⊕	- - -	+ + +	
Uninoculated					+ -

| | | |

(4) Fertilizer Trials on Alfalfa Fields

Eight trials were put on in the spring and summer of 1950, three near Edmonton, three just north of the town of Brooks, Alberta, in the Eastern Irrigation District, and two on the Magrath Irrigation District southwest of Magrath. The two latter trials were put on just after the first cutting of alfalfa had been removed. The plots were one rod square, laid out in the form of a Latin Square, and the fertilizer treatments were (1) check, (2) sodium molybdate, (3) 11-48-0 + sulphur + copper sulphate + manganous sulphate + zinc sulphate, and (4) sodium molybdate + all other treatments. For the trials at Magrath, a treatment of 11-48-0 alone was added. However, no significant differences were obtained between any of these treatments. Hay yields were very good on all of the irrigated trials, and any increase due to the fertilizers would have resulted in phenomenal total yields.

(4) Fertilizer Trials on Arable Fields

Eight trials were set up in the autumn and summer of 1970, three from December, before the end of the year, almost, to the Eastern Fertilizer Centres, and two of the regional fertilizer liaison assistant to Reports. The two latter trials were put on just after the first setting of nearly two dozen trials. The plots were two square, laid out in the form of a Latin Square, and the fertilizer treatments were (1) check, (2) urea ammonium (3) 11-44-0 + sulphur + copper sulphate + manganese sulphate + zinc sulphate, and (4) sulphur sulphate + all other treatments. The two trials at Reports, a research of 11-44-0 alone was added. However, no significant differences have obtained between any of these treatments. Any yields were very good on all of the fertilized trials, and any increase due to the fertilizers could have resulted in phenomenal total yields.

DISCUSSION OF RESULTS

The problems involved in the study of bacteriology as compared with those encountered in chemistry or physics may be compared to those of picking blueberries while wearing boxing gloves. When soil microbiology is being studied, the analogous problem becomes more complicated by the necessity of reaching at arms length under a wire fence for the berries. Thus it may be seen that the results obtained represent observations of the end products of the microbiological "reactions" rather than of the reactions themselves. The impossibility of dissecting the blueberries under the conditions specified may be readily seen, but the analogous condition for the bacterial reactions is less obvious. Once this is recognized, the results obtained in this investigation may be better understood.

(1) Experiments with Humus and Soil Cultures of Rhizobia

The effect of the addition of inorganic nutrients to the culture medium appears to depend largely upon the amounts of those nutrients present in the base originally. Where nutrients of several kinds were added to peat-black soil mixture, the increases obtained in numbers of rhizobia were much greater than increases obtained when the same nutrients were added to a peat-carbon black mixture, which

is an excellent base for the growth of rhizobia. Also, when phosphate was added to a brown soil low in this element, the numbers of bacteria were increased and maintained over a period of a year and a half. Sucrose, which alone of all the nutrients used, functions as an energy source for the bacteria, gave large increases in both bases, however.

The effect of nitrates and sucrose on the growth of rhizobia was extremely interesting. Hills (32) had found that the presence of 500 - 1000 ppm nitrate nitrogen in the soil culture medium depressed the growth of rhizobia, and it was thought that the presence of an easily available energy source might stimulate the growth of the bacteria. However, it was found that in the presence of all but the lowest concentrations of sucrose the growth of the bacteria was greatly repressed in cultures containing 500 ppm of nitrate nitrogen. Dilutions as low as one in five hundred (the dilution most commonly used in this work was one in five million) frequently failed to find any living rhizobia. No explanation has offered itself for this phenomenon, since the bacteria grow readily in the presence of much higher concentrations of sucrose alone. However, it seems apparent that it is important to control the level of nitrate nitrogen in the culture medium when making up commercial cultures.

Mineral oil and sodium alginate were added to the humus cultures to increase the numbers of bacteria which would stick to the seed when inoculation was done. Sodium

alginate was very sticky and appeared promising, but in the concentrations necessary for good sticking, the growth of the bacteria was much depressed. This was probably due to the excess of soluble materials introduced with the sodium alginate. After more than a year, possibly after the bacteria had consumed much of the soluble material, the numbers of organisms in these cultures rose very rapidly.

Mineral oil was less effective as a sticker, but offered less of an obstacle to the growth of the bacteria. Satisfactory numbers of rhizobia were maintained for considerable lengths of time in the presence of large quantities of mineral oil. The value of mineral oil as a preservative was somewhat disappointing. Its value in preserving cultures of rhizobia on agar slants for a period of several years had been proven beyond doubt. However, in the humus cultures, there was no evidence that it preserved alive larger numbers of bacteria or that the cultures dried out more slowly when oil was used.

The use of refrigeration in preserving humus cultures of root nodule bacteria appeared to give very promising results. In all cases, where cultures were refrigerated, larger numbers of the bacteria were preserved alive longer. Vass (75) showed that the rhizobia are not sensitive to cold, and the effect is probably partly at least the result of a simple slowing down of the metabolic

processes of the organism. If the cost of cold storage proves to be reasonable, this method promises to be very valuable in the handling of humus cultures of rhizobia.

Screw-top glass jars appear to be a very satisfactory substitute for paper cartons in marketing commercial cultures of rhizobia. Cost may be a limiting factor, but on the other hand, the earlier jelly cultures of rhizobia were marketed in glass bottles. The rhizobia survive for some time in the glass jars, which allow aeration without excessive moisture loss, and especially in conjunction with refrigeration, should prove to be extremely useful in marketing effective cultures of rhizobia. Contamination is a problem that must be considered, but it can probably be eliminated by proper handling and sterilization. It could not be worse than in the present paper cartons.

The problem of the condition of the bacteria in the cultures is an interesting one. The bacteria are non-spore formers, and yet it seems very unlikely that they are in a state of active growth. An attempt was made during the investigation to determine the respiratory activity of the organisms in humus cultures, both with the Lees respirometer and with a titration method. However, it proved to be extremely difficult. There was no apparent oxygen uptake by cultures in the Lees respirometer, even after several hours, using the untreated cultures, or

the cultures moistened with either distilled water or very dilute sucrose solution. Similarly the amount of carbon dioxide produced over a period of one to three days was very small. Hence it was concluded that the organisms were probably in some dormant state, as similar much older cultures were giving plate counts of several hundred millions per gram.

(2) Oxygen Uptake of Rh. Meliloti in Soils

This experiment was in the nature of a preliminary trial to obtain some idea of the possibilities of the Lees respirometer, as well as to gain information concerning the conditions influencing the survival of Rh. meliloti in irrigated soils. Surveys of the nodulation of pea and alfalfa fields in the irrigation districts of southern Alberta had indicated that conditions were sometimes such that nodulation of alfalfa was very poor. This experiment was intended to be the beginning of an investigation into possible causes of the poor nodulation.

The soils used were generally low in organic matter, except for soil number two. Thus organic materials were tried first to determine their effect on the growth of rhizobia in these soils, and to determine the length of time the effect, if any, would last in the presence of the general soil population.

When the determinations of oxygen uptake were made without any previous incubation of the soil-straw or soil-alfalfa mixture, the uptake of oxygen was very large, and the experiments could only be carried on for about twenty hours before the limit of measurement of the apparatus was reached. There was a much greater oxygen uptake where alfalfa was added. Mr. G. S. Cooper, of the Department of Plant Science, suggested that the amount of soluble carbohydrates in alfalfa was considerably higher than in wheat straw, and this excess was probably the reason for the increased oxygen uptake. The higher nitrogen content of the alfalfa very likely had some effect on the increased uptake also.

It was expected that the decomposition of the straw and alfalfa by the soil microflora would reduce the supply of energy sources easily available to the rhizobia, but in turn would break down the more resistant parts of the organic matter, and make a certain amount of soluble material available. This appears to have been the case. The very large oxygen uptakes first obtained were not repeated in determinations made after the soil had been incubated for 10 - 66 days. As the incubation time was increased, there was a progressively smaller increase in oxygen uptake, as the added organic matter was broken down, leaving the more resistant materials as the easily available ones were used up. In some cases, the oxygen uptake was greater where straw rather than alfalfa was added, after the soil had been incubated for a

fairly long period of time. This might be taken to mean that the straw was less readily broken down than the alfalfa at first, thus leaving more energy materials fairly easily available for a longer period of time. However, the evidence is not conclusive on this point, as the results were not consistent enough to be considered very significant.

The type of soil and possibly its previous handling had some effect on the results obtained. Oxygen uptake was highest for the longest time in soil number six, a heavy soil which has been farmed very well for a good many years. Uptake was lowest in soil number three, a much lighter soil, also farmed intensively for about fifty years.

Steam sterilization of the soil undoubtedly affected the results obtained. In previous unpublished experiments on the oxygen uptake of the unsterilized soils, soil number two showed a definitely greater uptake than did the others. Yet when steam sterilized in the presence of fresh organic matter, this soil had the lowest oxygen uptake of all. Simpson (59), Malowany, as reviewed by Simpson, and many other workers found that heat sterilization of the soil produced chemical changes in organic matter. It is probable that under some conditions toxic materials are produced. Sterilization by some other means, such as the use of ultrasonic methods or of non-persistent organic agents, if it could be accomplished without producing as great changes in the soil, would improve the results.

The general form of the curves obtained indicates that after about two days the oxygen uptake by the bacteria drops to a very small amount. Plate counts on the soils at this stage would certainly show the presence of extremely large numbers of microorganisms. This is another indication that rhizobia in the soil are probably in a state of dormancy or semi-dormancy a good deal of the time.

(3) Dilution Counts of Rhizobia in Soils

If the numbers of rhizobia in soil were low, nodulation would of course be poor. It was found by Wilson (80, 81) and Thorne and Brown (63) that in Iowa the numbers of root nodule bacteria in the soils where the appropriate legumes were grown were in the neighborhood of hundreds of thousands per gram. Accordingly counts were made on soils from the fertilizer trial alfalfa plots near Edmonton, and on a sample from the University Soils farm alfalfa plots, where nodulation of alfalfa had been very poor for two or three years. However, it was found that Rh. meliloti were as numerous in two of these soils as in the soils studied by Wilson (80, 81) and by Thorne and Brown (63), and in only one soil were they rather scarce. It is probable that the very dry growing seasons affected the nodulation, rather than the lack of rhizobia in the soil.

(4) Fertilizer Trials on Alfalfa Fields

No effect was observed on application of fertilizers to alfalfa plots. In the Edmonton area this was probably because of the dry summer. In the irrigated areas, it is probable that some other factor or factors influenced the results obtained, as the soils of both the Brooks and Magrath areas are known to be low in phosphorus. It is very likely that no minor element deficiency existed, but the lack of yield increase when phosphate was applied was very likely because of phosphate fixation. The fertilizers were of necessity applied broadcast, and it has been found in Alberta and elsewhere that it is usually necessary to drill in phosphate fertilizer at seeding time to obtain any appreciable results. This was probably the reason for the lack of effects observed.

SUMMARY

(1) A study was made, first, of several factors influencing the growth and survival of rhizobia in humus and soil cultures, and second, of the oxygen uptake of Rh. meliloti in sterilized soils to which organic materials had been added.

(2) The moisture content of the cultures was important; as water was lost, the numbers of rhizobia decreased.

(3) The addition of sucrose to the humus cultures ordinarily produced large increases in the numbers of these bacteria. The addition of phosphorus, potassium or calcium carbonate also seemed to increase the numbers of organisms present, but the increase was less marked.

(4) The addition of sodium nitrate alone to the cultures appeared to stimulate the growth of the rhizobia. The addition of sucrose as well as nitrates produced a very large drop in the numbers of these bacteria.

(5) Mineral oil did not appear to depress the numbers of rhizobia in the humus cultures to any great extent.

(6) Sodium alginate inhibited the growth of these

bacteria if added to the medium in quantities large enough to affect the physical properties of the medium noticeably.

(7) Screw-top glass jars for packaging commercial legume inoculants appeared to be an improvement over the paper cartons now in use.

(8) Refrigeration of the cultures was an excellent method of maintaining large numbers of living bacteria for a long time.

(9) The water loss from refrigerated cultures was considerably less than from cultures held at room temperatures.

(10) Oxygen uptake of Rh. meliloti in soil is discussed. The type of organic matter added to the soil, the texture and previous history of the soil and the method of soil sterilization all appeared to influence the oxygen uptake.

(11) Dilution counts of Rh. meliloti in four field soils showed a fairly wide variation in numbers of bacteria in soils from the Edmonton area.

(12) Fertilizer trials on several Alberta alfalfa fields showed no increases in yield. Possible reasons for these results are suggested.

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1. [illegible] . . . [illegible] . . . [illegible] . . .

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